

BIOCHEMICAL STUDIES
OF
THE NORMAL HUMAN BREAST

by

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This thesis is dedicated

to

my mother, Yap Swee Tin

and

my late father, Yap Pow Veng

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Declaration

Except where acknowledgement is made by reference, the experiments described in this thesis were the unaided work of the author.

While registered as a candidate for the degree of Doctor of Philosophy, the author was not a registered candidate for another award of the CNAA, or of a University.

The author attended the Seminars of the MRC Reproductive Biology Unit and the Department of Obstetrics and Gynaecology, as well as Meetings of the University of Edinburgh Department of Clinical Surgery Breast Group in connection with the programme of research and in partial fulfilment of the requirements for the degree of Doctor of Philosophy. In addition, the author attended the Meetings, Seminars and Courses of the Edinburgh Metchnikoff Club, the Seminars of the Edinburgh and S.E. Scotland Regional Blood Transfusion Service and the Radiation Protection Course organised by the University of Edinburgh.

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ABSTRACT

BIOCHEMICAL STUDIES OF THE NORMAL HUMAN BREAST

by

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The age of the mother at the birth of her first full-term infant determines the subsequent risk of developing carcinoma of the breast throughout her life. In this thesis, the hormonal control of the normal breast was studied so as to achieve a better understanding of the aetiology of breast carcinoma. Oestrogen receptor concentrations were studied, and low but detectable levels were found in normal human breast tissue. However, the assay used was insufficiently sensitive to detect variations between breast tissue from nulliparous and parous women.

Large quantities of secretory IgA (11S IgA) are synthesised in the breast during post-partum lactation, and therefore mammary IgA production was measured in different reproductive states. Neonatal milk ('witch's milk'), galactorrhoeic secretions due to hyperprolactinaemia, normal breast secretions and cyst fluid from patients with gross cystic disease of the breast were examined for IgA, the latter because cyst fluid might reflect IgA synthesis by surrounding breast tissue. An IgA radioimmunoassay was developed for the small quantities of secretion available and methods were also developed for differentiating 11S IgA from serum IgA (7S IgA).

Low but detectable concentrations of 11S IgA were found in neonatal milk reflecting the immaturity of IgA synthesis in the neonate. Galactorrhoeic mammary secretions, and normal breast secretions from non-lactating nulliparous and parous women were found to contain concentrations of 11S IgA comparable to colostrum and milk, suggesting that mammary 11S IgA synthesis is not uniquely associated with the end of a full-term pregnancy. Cyst fluids were found to contain either 7S IgA, 11S IgA or mixtures of both, suggesting that there may be different modes of cyst formation.

Finally, because colostrum and milk IgA provide immunological protection to the neonate, experiments were undertaken to discover if breast feeding altered neonatal serum IgA concentrations. No alterations were found, suggesting that IgA in the breast fed neonate acts only in the intestinal lumen.

CHAPTER 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

The distinguishing feature of all species in the class Mammalia is the possession of mammary glands. These glands occur in both sexes, and may be of variable size, shape and number. In the human, the breast (a term whose usage will be restricted in this thesis to the mammary gland of Homo sapiens alone) has multiple functions: it provides immunological protection and nutrition to the neonate in the form of colostrum and milk (reviewed by Jelliffe and Jelliffe, 1978). In addition, the breast has a subsidiary function as an organ of sexual attraction (Ford and Beach, 1952), and the highly innervated nipple (Robinson and Short, 1977) acts as an important sensory receptor of suckling by the neonate. This suckling stimulus initiates a reflex discharge of oxytocin and prolactin from the pituitary gland (Tyson, Friesen and Anderson, 1972). Oxytocin stimulates the initial flow of milk and prolactin ensures lactation is sustained. This suckling stimulus also inhibits maternal pituitary gonadotrophin secretion (Baird, McNeilly, Sawers and Sharpe, 1979), and lactational amenorrhoea ensures adequate natural spacing between successive births (Short, 1976).

The Development of the Breast

The development of the breast (Tholen, 1949; Dabelow, 1957; Patten, 1968 and Raynaud, 1961) occurs early in embryonic life, and a mammary band can be discerned in embryos 4 mm. in length. This mammary band develops into a primitive mammary bud by about two months gestation, but little further growth occurs till about the fifth month of gestation. At this time, the mammary bud, consisting of a solid, spheroidal clump of cells, begins to form 15 to 20 secondary buds which eventually form the basis for the duct system of the mature mammary gland (Fig. 1.1). These secondary buds lengthen, and this

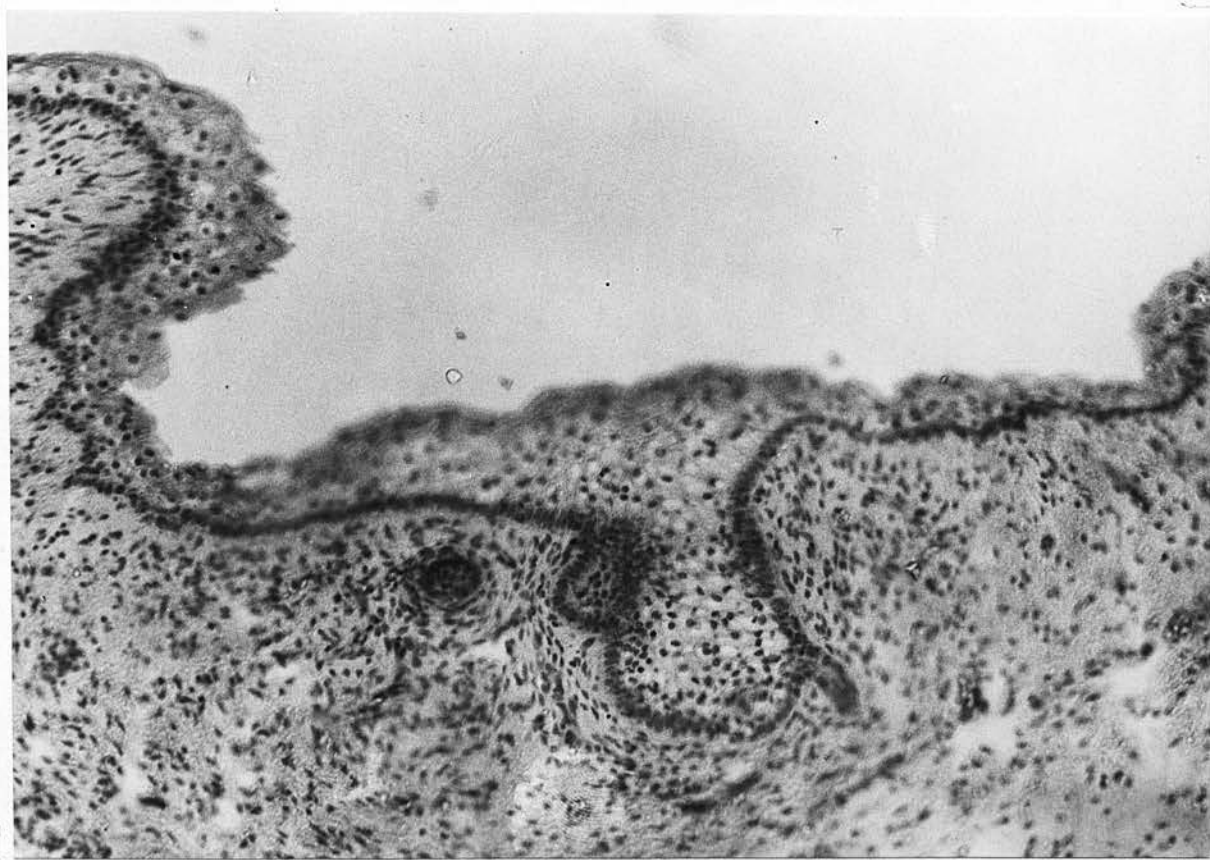
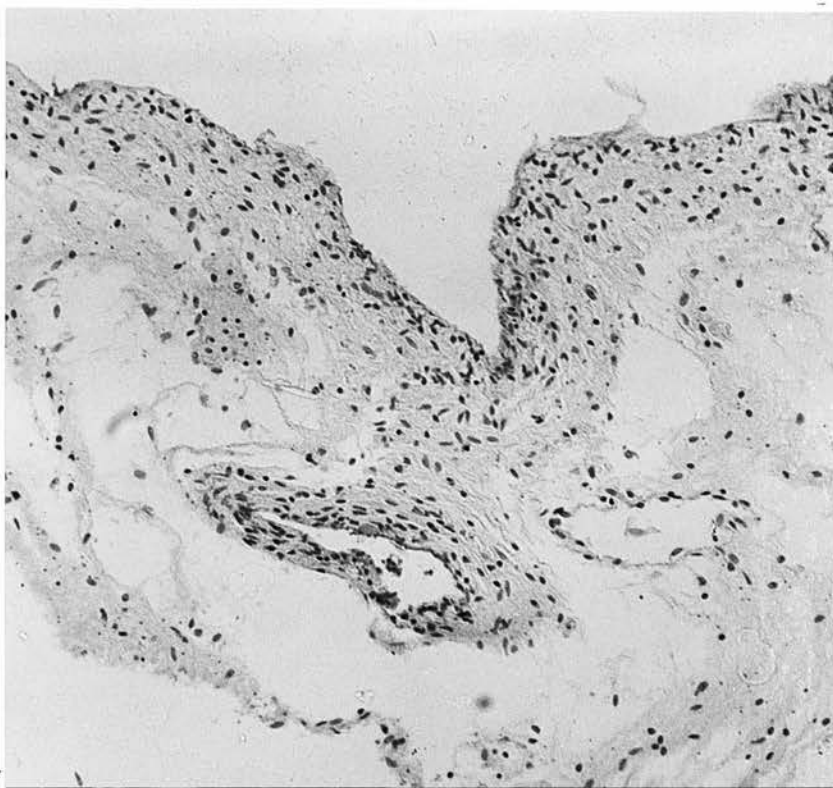


Fig. 1.1 Developing mammary tissue in a human foetus of 8-10 weeks gestation (above) and 16-18 weeks gestation (below). Foetal time was obtained by courtesy of Dr. C. Gosden (x 1000).

ingrowth of ectoderm protrudes into the underlying mesenchyme, forming cylindrical cords. As pregnancy advances, these cylindrical cords bifurcate successively, and lumina develop surrounded by a wall composed of two concentric layers of cuboidal cells. The inner layer of cells eventually forms the secretory epithelium, and the outer layer the myoepithelium. The ends of the cords develop eventually into glandular acini.

The factors that control this developmental process are not known, but may involve some form of epithelial-mesenchymal interaction. In the mouse, it has been shown that the stromal tissue of the mammary gland has an important regulatory action on ductal growth (Faulkin and de Ome, 1960) and the epithelial necrosis that takes place during the twelfth to fourteenth day of gestation in the male foetus is secondary to the action of testosterone on the mesenchyme (Kratochwil and Schwartz, 1976). It should be noted however that in humans, no such male-specific epithelial necrosis takes place, and hypertrophy of the breast (see below) takes place in a large proportion of neonates, regardless of sex (Lyons, 1937; Bluestein and Gardner, 1966; Dr. J. McKiernan, personal communication).

Shortly before parturition, the embryonic mammary ducts undergo a limited degree of proliferation (Dabelow, 1957), which is thought to be due to the influence of maternal hormones circulating in the foetal blood at that time (Mayer and Klein, 1961). This limited degree of proliferation manifests itself in the engorged breasts observed during the immediate post-partum period. In addition, a milk-like secretion (occasionally called 'witch's milk') can be expressed from some neonatal breasts (Bluestein and Gardner, 1966), for a period lasting up to twenty days (Ponzzone and Voglino, 1976).

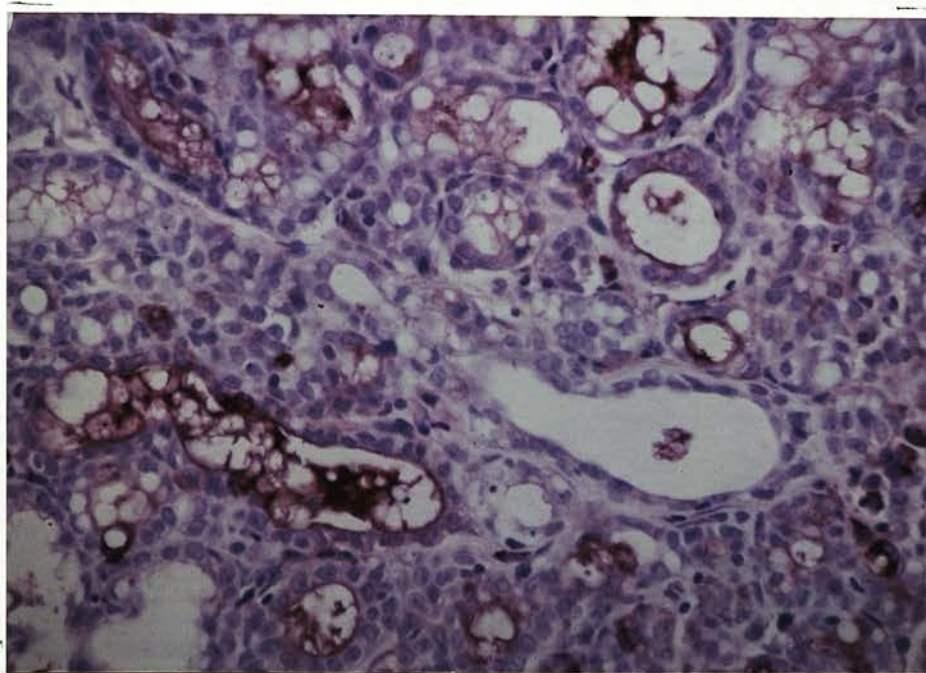


Fig 1.1 (cont.) Mammary tissue in a non-lactating woman, showing a duct surrounded by featureless stroma (above, x 40) and in a lactating woman, showing proliferation of glandular tissue (below). A few IgA plasma cells are present, and are stained dark brown. (Immunoperoxidase preparation by courtesy of Dr. R.S.H. Pumphrey).

Gradual involution of the neonatal breast occurs after birth, and from then on till puberty, the breasts of both sexes grow proportionately with the body as a whole. However, at the beginning of puberty, enlargement of the breast occurs in the female (Marshall and Tanner, 1969) which is thought to be due to increased ovarian oestrogen synthesis (Short and Drife, 1977). Following puberty, no further major changes take place in the breasts, but if pregnancy ensues, additional growth of the breasts and nipples takes place. The major changes taking place during pregnancy are the development of the stroma (consisting of fat and connective tissue), and of the alveoli. Branching of the ducts to form lobules occurs, and these changes are thought to occur in response to the raised circulating blood levels of oestrogen, progesterone, prolactin and placental lactogen (reviewed by McNeilly, 1977). Following the delivery of the baby and the placenta, a small amount of colostrum may be expressed from the maternal breast and is followed a day or two later by the secretion of milk. Lactation continues for a variable period, depending on the persistence of breast feeding and blood prolactin concentrations, but eventually milk secretion ceases, involution of the breast occurs, and no further developments take place till the next pregnancy, or the arrival of the menopause, when permanent involution takes place.

In nulliparous women, or in parous women following the end of lactation, but before the next pregnancy, the term "resting breast" is often used to describe the mammary gland that is not secreting milk. However, the breast of a non-pregnant woman is capable of responding to the action of steroid hormones, as shown by the changes in breast volume during the normal menstrual cycle, that have been ascribed to increased oestradiol and progesterone secretion in the luteal phase (Milligan, Drife and Short, 1975). In addition, cyclic variation of

DNA synthesis in human breast epithelium (Masters, Drife and Scarisbrick, 1977) and of IgA synthesis in parous (but not nulliparous) women has been reported (Drife, McClelland, Pryde, Roberts and Smith, 1976). Small amounts of secretion can be aspirated from at least 75% of non-lactating breasts (Petrakis, Mason, Lee, Sugimoto, Powson and Catchpool, 1975), also suggesting that the term "resting" breast may be a misnomer. In describing the non-lactating breast, it is worth noting that among mammals, human beings are unique in the relatively large size of their mammary glands outwith pregnancy and lactation.

Immunological Aspects of the Breast

The immunological protection provided by human milk has been recognised for decades (Woodbury, 1922; Grulee, 1934). Numerous studies have shown that the incidence of gastrointestinal infection is much lower among breast-fed infants (reviewed by Chandra, 1978) and one of the major antimicrobial factors in milk is secretory IgA (11S IgA). This immunoglobulin is found in very high concentrations in comparison with IgG and IgM (Hanson, 1961; McClelland, McGrath and Samson, 1978) and is synthesised by mammary sub-epithelial plasma cells (Lamm, 1976) that originate in the Peyer's patches of the gut (Walker and Isselbacher, 1977). Evidence for the entero-mammary circulation of lymphocytes in the human is based upon the observation of IgA antibodies in milk with a specificity for bacterial antigens commonly found in the intestine or other antigens deliberately instilled there (Adinolfi, Glynn, Lindsay and Milne, 1966; Allardyce, Shearman, McClelland, Marwick, Simpson and Laidlaw, 1974; Montgomery, Rosner and Cohn, 1974; Goldblum, Ahlstedt, Carlsson, Hanson, Jodal, Lidin-Janson and Sohl-Akerlund, 1975; Stoliar, Kaniecki-Green, Pelley, Klaus and Carpenter, 1976). This 11S IgA differs from the IgA present in serum (7S IgA) in being a dimer with an additional

protein component, secretory component, which confers on 11S IgA increased resistance to proteolytic digestion in the neonatal gut (Brown, Newcomb and Ishizaka, 1970). The exact mechanism by which 11S IgA acts is not known, but it is capable of causing the agglutination of a wide range of enteric organisms (McClelland, Samson, Parkin and Shearman, 1972), and thereby reducing the ability of an organism to colonise the intestine, possibly enhancing its clearance by intestinal peristalsis. Secretory IgA acts with lysozyme to lyse E. coli (Adinolfi et al., 1966; Burdon, 1973) by activating the complement sequence, presumably by the alternative pathway (Götze and Muller-Eberhard, 1971). Other antimicrobial factors are also present in colostrum and milk such as lysozyme (Adinolfi et al., 1966), lactoferrin (Bullen, Rogers and Leigh, 1972), complement (Adinolfi et al., 1966), lactoperoxidase (Reiter, Marshall, Bjorck and Rosen, 1976) and Bifidus factor, which promotes the growth of lactobacilli (Chandra, 1978).

Colostrum and milk also contain various cells, including lymphocytes which are capable of blastoid transformation on exposure to the polyclonal mitogen, phythaemagglutinin (Smith and Goldman, 1968) as well as the synthesis of immunoglobulins and complement (Murillo and Goldman, 1970). However, the role of the cellular constituents of colostrum and milk in relation to the immunological protection of the neonatal gut is not clearly delineated.

Breast Histology and the Menstrual Cycle

From the preceding description of the development of the breast, it can be seen that the breast is a complex organ composed of different tissues: namely epithelium (consisting of mammary ducts and glandular acini) lying within mesenchyme (consisting of connective tissue stroma containing the vasculature and adipose tissue). In addition there are various cells types like plasma cells and T lymphocytes that infiltrate

the breast. The proportion of some of these cells and of the secretory epithelium, may depend on whether the breast is "resting", lactating, or in a post-lactational state (Pumphrey, 1977). There have been a number of studies which have attempted to correlate histological changes in the breast to the phase of the menstrual cycle. Rosenberg (1922) claimed that budding of ductal epithelium occurred in the pre-menstrual phase, with the subsequent disappearance of the acini formed from this proliferation after menstruation had occurred. This was disputed by Dieckmann (1925) who reanalysed Rosenberg's material and concluded that the differences detected were due to the patient's age rather than the phase of the menstrual cycle. In another study, Foote and Stewart (1945) claimed that the number and size of the alveoli increased prior to menstruation and that the number of lobules increased with age. In a comprehensive study by Dabelow (1957), the changes in the breast during the menstrual cycle were found to involve different portions of the mammary gland to varying degrees, and some portions of the mammary gland were not affected at all. Two recent studies have confirmed the histological heterogeneity of the breast (Wilson, 1976; Short and Drife, 1977). For instance, in the same specimen of breast tissue, the proportion of glandular tissue varied at random from 0.3% to 29.0% of the area of the section and none of the histological variables examined showed consistent variations with the stage of the menstrual cycle in either nulliparous or parous women (Short and Drife, 1977). Histological studies of the normal non-lactating breast are therefore fraught with difficulties, and any conclusions reported must be treated with caution.

Carcinoma of the Breast

In the preceding sections of this chapter, only the normal lactating or non-lactating breast has been considered. However, the

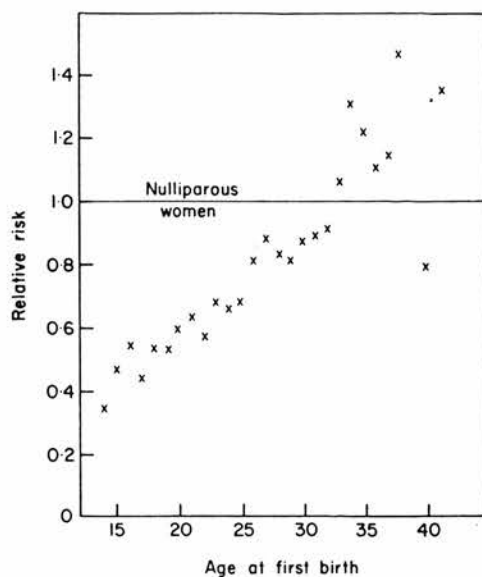


Fig. 1.2: The relative risk of developing breast cancer in relation to the mother's age at the birth of her first child. The risk in nulliparous women is taken as one. Pregnancy before the age of 35 reduces the risk, and after 35 increases it. From MacMahon, Cole & Brown (1973).

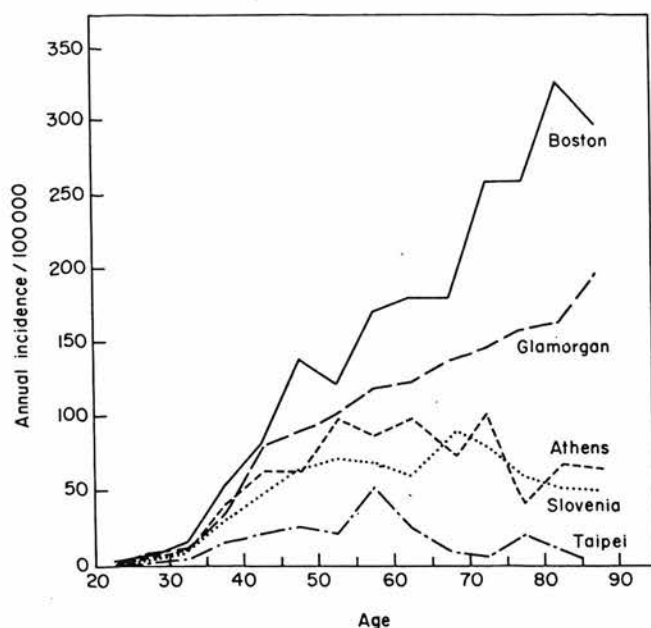


Fig. 1.3: Age-specific incidence rates of breast cancer in five different areas of the world. From MacMahon, et al. (1973).

major impetus for research into the breast is the occurrence of carcinoma of the breast. Among Western women, this is the commonest cancer, and the leading cause of death from malignancy (Registrar General, Scotland, 1976). It affects approximately one woman in twenty, and despite extensive research into the aetiology of the disease, the death rate has remained constant (Myers, 1972). In an attempt to discover the 'cause' of breast cancer, various risk factors have been elucidated of which the most important is the age at which a woman bears her first full-term child (MacMahon, Cole, Lin, Lowe, Mirra, Ravnihar, Salber, Valaoras and Yuasa, 1970 b; Table 1-1). Figure 1.2 clearly illustrates the finding that the younger the maternal age at first pregnancy, the lower the risk of developing breast cancer. This finding, together with observed increased risk associated with an early menarche, or a late menopause, led Short and Drife (1977) to postulate that the nulliparous breast might be stressed by a repeated succession of menstrual cycles, whereas following the first full term pregnancy, a structural or functional change in the breast might occur, protecting the breast from subsequent malignant change.

Lactation itself has little, if any, effect on breast cancer risk according to MacMahon, Lin, Lowe, / although this was disputed by Ing, Mirra, Ravnihar, Salber, Trichopoulos, Valaoras & Yuasa (1970a), Ho and Petrakis (1977) in their retrospective study of Hong Kong women who breast fed unilaterally only (see below). The decreased risk observed with multiparity can be accounted for by the association of this factor with an early first pregnancy (MacMahon et al., 1970b). The increased risk of developing breast cancer seen in patients with pre-existing fibrocystic disease of the breast is thought to be due to an unknown predisposing factor shared by the two diseases, rather than a causal association (Haagensen, 1971).

Table 1-1

Major risk factors for breast cancer in women

<u>Factor</u>	<u>Increased risk</u>	<u>Decreased risk</u>
Geographic/ethnic	Western European North American	Japanese, Taiwanese, South African (blacks) Indians
Age at first birth	After age 30	Before age 20
Parity	Nulliparity	Multiparity
Age at menarche	Before age 13	After age 16
Age at natural menopause	After age 50	Before age 45
Ovariectomy	None	Before age 45
Nutritional	Obesity	None
Ionising radiation	Ionising radiation	None
Familial	Positive family history of breast cancer	None
Previous cancer	Breast, endometrium, colon-rectum, salivary gland, cancer	Cervix
Prior benign breast disease	Fibrocystic breast disease	None

(From Levin and Thomas, 1977)

What then might be the mechanism of protection against developing breast cancer that results from a full term pregnancy? One possibility is that some substance or cell type is present in the breast towards the end of pregnancy and that this somehow confers the decreased risk observed. An attractive candidate for this hypothesis might be some component of the immune system as it is known that in rare primary immunodeficiency states, the frequency of malignancy is approximately 10,000 times greater than that in the general age-matched population (Gatti & Good, 1971). In patients with common variable (late onset) acquired hypogammaglobulinaemia, malignancy occurs much more frequently than in the normal population (reviewed by Ross and Asquith, 1979). In secondary immunodeficiency (following immunosuppressive therapy for renal transplantation), de novo cancers occur at approximately 100 times the frequency observed for the general age-matched population. In one series (Penn, 1974), epithelial tumours accounted for 67% of the cancers, an interesting finding in comparison with cancer of the breast where almost all cancers are epithelial in origin. As described above, large numbers of lymphocytes enter the breast towards the end of pregnancy (Pumphrey, 1977), and some of these lymphocytes secrete IgA, and smaller quantities of IgG and IgM. Other lymphocytes occur in colostrum and milk, suggesting that the involvement of the immune system at the end of a full-term pregnancy might provide a possible explanation for the protective effect observed.

Another possibility that might also explain the protective effect of an early full term pregnancy might be the removal at the end of pregnancy of some potentially carcinogenic substance, or the removal of a clone of cells likely to undergo subsequent malignant transformation. A variety of ingested and inhaled chemical substances have been demonstrated in the secretions obtained from non-lactating women, and

among these substances may be initiating and promoting factors acting on the epithelium to produce malignant change (Petrakis, 1977). Ing et al. (1977) reported the intriguing finding that in a group of Chinese women in Hong Kong who traditionally only fed their babies from one breast, cancer developed only in the unsuckled breast of 79% of such women when postmenopausal, a distribution that was significantly different from that seen in the normal Chinese population in Hong Kong. This proposal that the mammary ducts contained potentially carcinogenic substances would be in accord with the proposal by Short and Drife (1977) that a repeated succession of menstrual cycles might be harmful, as each cycle might lead to an increase in the quantity of carcinogenic substances within the mammary ducts. Alternatively, the death of ductal and alveolar cells at the end of lactation, associated with engorgement and milk stasis regression, would reduce the overall number of potentially malignant cells.

A third possibility is that some change in endogenous hormone secretion occurs associated with a full term pregnancy, and that this confers a permanent change in either the breast itself, or in the subsequent pattern of hormone secretion. Among the endogenous hormones, it is probable that oestrogens are involved in the genesis of breast cancer, as it has been shown that ovariectomy prior to the menopause decreases the risk of developing breast cancer and the earlier ovariectomy is performed, the greater the decrease in risk (Hirayama and Wynder, 1962; Feinleib, 1968). The protection against developing breast cancer conferred by a late menarche and an early natural or artificial menopause could be linked with an alteration in oestrogen synthesis by the ovary.

The relationship of oestrogens to breast cancer can also be studied in women who receive exogenous oestrogens over a long period,

either for contraceptive purposes, or for menopausal symptoms, or after a hysterectomy. Epidemiological studies in this area are fraught with difficulties, due to alterations in patterns of oral contraceptive or hormone replacement usage. In addition, the wide variety of preparations used, and the requirement for case control studies of an adequate size or cohort studies of a sufficiently long period contribute to difficulties of data interpretation. To date, on the basis of cohort or case-control studies, (reviewed by Vessey, 1977), there appears to be no clear evidence that oral contraceptives are linked with breast cancer. However, in the case of oestrogens administered after the menopause, or after hysterectomy, evidence from two studies (Burch, Byrd and Vaughn, 1976; Hoover, Gray, Cole and MacMahon, 1976) considered to be carefully performed (Vessey, 1977) indicates that the risk of breast cancer might be slightly increased by the administration of oestrogens as hormone replacement therapy.

The problem with all hypotheses linking oestrogen secretion with breast cancer, as pointed out by MacMahon, ^{Cole and Brown} (1973) is that they fail to explain why the protective effect should only be restricted to the first pregnancy. Even the oestriol hypothesis, popularised by Cole and MacMahon (1969), which included in it an explanation for the protective effect of the first pregnancy, is no longer considered to be valid (Kirschner, 1977). When considering other endogenous hormones (e.g. androgens, progesterone, prolactin and thyroid hormones) the same problem arises as with oestrogens: that it is difficult to formulate a hypothesis connecting a particular hormone with the restriction of the protective effect to the first pregnancy. It is for instance difficult to reconcile the finding that breast cancer patients had a higher incidence of anovulatory cycles than normal women (Grattarola, 1964) and that inadequate corpus

luteum function may be very common among breast cancer patients (Sherman and Korenman, 1974) with the restriction of the protective effect, unless a full-term pregnancy somehow alters the pattern of pituitary hormone secretion.

Another important epidemiologic observation has been the striking variation in breast cancer incidence among different areas of the world. The incidence is five to six times higher in North America and Northern Europe than in most areas of Asia and Africa. Striking differences also occur in age incidence patterns (Fig. 1.3) and it is interesting to note that differences in reproductive practice are unlikely to be responsible for the major international differences in breast cancer rates (MacMahon et al., 1973). Numerous hypotheses have been advanced to explain this geographical variation, including diet, histologic type of the tumour and genetic differences (reviewed by MacMahon et al., 1973) but at the present time, no definitive explanation is available.

Animal Models for Breast Cancer

A number of animal models have been used experimentally to increase our understanding of breast cancer. The species with the highest spontaneous incidence of mammary cancer are man, the laboratory rat and mouse, and the domestic dog and cat. However, as pointed out by Short and Drife (1977), only in the human does mammary development occur at puberty, whereas in the cat, rat and mouse, mammary development and lactation are dependant upon copulation. In the dog, mammary development and lactation occurs at puberty, and are independant of pregnancy. Thus, it is possible that extrapolation of experimental findings about mammary carcinogenesis in animals to breast cancer in humans may be unwarranted. However, it is of interest to note that ovariectomy, or absence of pregnancy is a major aetiological factor

in the development of mammary in the cat (Dorn, Taylor, Schneider, Hibbard and Klauber, 1968; Weijer, Head, Misdorp and Hampe, 1972), and in the rat (Howell and Mandl, 1961). In the dog, the evidence is less conclusive that pregnancy is related to mammary carcinoma incidence (reviewed by Hamilton, 1974).

Differences also exist between species in immunological function of the mammary glands. This is probably related to the fact that in some mammals, for example humans, both the placenta and the mammary gland are involved in the transfer of immunoglobulin to the foetus and the neonate. In other mammals, e.g. cows, goats and sheep, only the mammary gland is involved in the transfer of immunity. A third group which includes cats, dogs and rodents is intermediate between the two groups described above. This difference is reflected in the mammary gland. In cattle, the acinar epithelial cells of the mammary gland possess specific receptors for the Fc portion of the IgG molecule. Selective transport occurs of a single subclass, IgG₁, from serum to colostrum, and the IgG₁ content in colostrum is 84% of the total immunoglobulin, whereas IgG₂ is 5% only. IgA and IgM are also present in low concentrations in cow's colostrum (7% and 5% respectively). By contrast, in human colostrum, IgA predominates, constituting 98% of the total immunoglobulin. IgG (0.5%) and IgM (2%) are much lower, reflecting the fact that most of the immunoglobulin in human colostrum and milk is synthesised in the breast by plasma cells, the majority of which secrete IgA (McClelland et al., 1978; Butler, 1979). The rat occupies an intermediate position with IgA and IgG constituting 63 and 36% of colostrum immunoglobulin respectively. This data may be of significance when one considers that mammary cancer is virtually unknown in the cow, despite the selection for large udder size and high milk yield for many generations (Short and Drife, 1977).

As well as using animal models, many experimental studies attempting to unravel some of the factors that might be related to mammary carcinogenesis have utilised human breast tumours or tissue from patients with existing or previous breast carcinoma. However, it has been shown that the period between exposure to a carcinogen, and the development of tumours of the respiratory tract can range from 10 to 30 years (Hueper, 1966) and it is likely that a latent period of similar magnitude exists for breast cancer. Furthermore, it has been proposed (on the basis of tumour size doubling times) that a malignant tumour of the breast may exist for ten years prior to clinical detection (Gullino, 1977). It is therefore important to study normal i.e. non-malignant breast tissue obtained from female patients who have no existing or previous breast malignancy if we are to understand why malignant change occurs in the female breast.

Aims of the thesis

The aim of the experiments described in this thesis was to study the hormonal control of the normal breast so as to achieve a better understanding of the aetiology of carcinoma of the breast in humans. An oestrogen receptor assay was therefore validated, and the oestrogen receptor concentration in normal human breast measured. In addition, IgA production was measured in different reproductive states. Neonatal milk ('witch's milk'), galactorrhoeic secretions due to hyperprolactinaemia, and normal breast secretions were examined for IgA. Cyst fluid obtained from patients with gross cystic disease of the breast was also examined for IgA, as the fluid might reflect the IgA synthetic activity of the surrounding breast tissue. A radioimmunoassay for IgA was developed for the above studies because of the small quantities of secretion available and methods were developed for differentiating 11S IgA from 7S IgA. The radioimmunoassay was also used to assess if

quantitative changes in IgA synthesis occurred during the menstrual cycle, as earlier studies by Drife et al. (1976) had demonstrated that qualitative changes occurred during the menstrual cycle in IgA synthesis in parous but not nulliparous women.

Finally, because of the importance of colostral and milk IgA in the immunological protection of the neonate, experiments were undertaken to discover if breast feeding produced a detectable effect on serum IgA concentrations in the neonate.

CHAPTER 2

OESTROGEN RECEPTOR CONCENTRATIONS IN

RAT AND HUMAN TISSUES

CHAPTER 2 OESTROGEN RECEPTOR CONCENTRATIONS IN RAT AND
HUMAN TISSUES

2.1 INTRODUCTION

The normal human female breast is a target organ for steroid and protein hormone action, as described in Chapter 1 (Marshall and Tanner, 1969; Milligan et al., 1975; Drife et al., 1976; Masters et al., 1977; McNeilly, 1977; Short and Drife, 1977). The initial event in the mechanism of action of the steroid oestradiol- 17β in a target organ like the uterus is the binding of the steroid to a specific, cytoplasmic, soluble protein, the oestrogen receptor (Toft and Gorski, 1966). Thus the receptor may exist either in a free state or bound to oestradiol- 17β . Most reports concerning cytoplasmic oestrogen receptor concentrations in rat and human tissues have examined only concentrations of free receptor, and it is possible that the failure of previous attempts to measure oestrogen receptors in normal breast tissue (Feherty, Farrer-Brown and Kellie, 1971; Leclercq, Heuson, Deboel and Mattheiem, 1975) was due to this.

The possibility of measuring both free and bound receptor by exchange assay has recently been demonstrated by Chamness, Huff and McGuire (1975); the method involves precipitating the oestrogen receptor with protamine sulphate (based on Steggles and King, 1970). In view of the fact that the other target organs for oestrogen action such as the uterus, vagina and hypothalamus have all been shown to contain a specific oestrogen receptor protein, an attempt was made to demonstrate the occurrence of oestrogen receptors in the normal breast as a preliminary step towards measuring changes in oestrogen receptor concentrations in response to the menstrual cycle and pregnancy.

An evaluation of saturation analysis and exchange assay using protamine sulphate precipitation was therefore undertaken by studying vacant and occupied receptor concentrations in rat uterus throughout the oestrous cycle (where fluctuations in plasma levels of oestradiol-17 β occur, resulting in possible differences in the proportion of free and bound cytoplasmic oestrogen receptor sites) and in other rat tissues. Saturation analysis and exchange assay was then used to study oestrogen binding in normal human breast tissue, human uterus, muscle and serum.

2.2 MATERIALS AND METHODS

Animals Vaginal smears were taken daily at 1000h from female Liverpool Hooded rats (60-90 days old) known to show regular 4-day oestrous cycles (Yoshinaga, Hawkins and Stocker, 1969). Between 1130h and 1230h on particular days of the cycle rats were killed by exsanguination through the aorta under ether anaesthesia. Uteri and samples of sciatic muscle were excised, trimmed free of fat and blood vessels, washed in Tris buffer (0.01 mol/l Tris-HCl, 1.5 mmol/l EDTA, pH 7.4 at 4°C) and stored at -70°C. Plasma was separated from blood before estimation of the concentration of oestradiol-17 β . Inguinal mammary glands were obtained from lactating rats of the same strain which were killed by cervical dislocation on day 21 post partum. Serum was obtained from additional non-pregnant female rats for the determination of the binding of oestradiol-17 β to serum.

Patients Normal mammary tissue was obtained from 13 pre-menopausal patients aged 16-43 years at the time of reduction mammoplasty or biopsy for benign breast disease. Representative sections were submitted to histology and it was demonstrated that the tissue was normal. Uterine tissue (predominantly myometrium) was obtained from four patients

undergoing hysterectomy and inferior rectus muscle from 4 patients undergoing cholecystectomy. Serum was also obtained from each of 6 female pre-menopausal subjects.

Radiochemicals 2,4,6,7-(n)³H - Oestradiol-17 β (sp.act.87-115 Ci/mol) was obtained from the Radiochemical Centre, Amersham and purified at intervals of 2 weeks by the method of Mikhail, Wu, Ferin and Vande Wiele (1971).

Determination of protein concentration The concentration of soluble protein in tissue homogenates or blood sera was determined by the method of Bradford (1976) using a bovine serum albumin standard.

Determination of plasma hormone levels Concentrations of oestradiol-17 β in rat sera were determined by Dr. R.A. Hawkins using the method of Hawkins, Freedman, Marshall and Killen (1975). The assay sensitivity for plasma oestradiol-17 β (as defined by Hawkins et al., 1975) was 13.5, 9.0 and 5.9 pmol/l for 1.5, 2.0 and 3.0 ml plasma respectively.

Determination of oestrogen binding site concentration:

Preparation of cytosol and sera The assay used was a modification of the method described by Chamness et al. (1975). Rat uterus, rat muscle, mammary tissue from lactating rats, human uterus, human muscle or normal human breast tissue were frozen with liquid N₂, pulverized in a stainless steel mortar and homogenized at 4°C in Tris-dithiothreitol buffer (0.01 mol/l Tris-HCl, 1.5 mmol/l EDTA, 0.5 mmol/l dithiothreitol, pH 7.4) using a Silverson homogenizer at maximum speed in two bursts of 10s each with a 15s cooling period between. Tissue homogenates prepared in this way and blood sera were centrifuged at 105,000xg to remove nuclei and cell debris: the resulting supernatants were incubated with dextran-coated charcoal, centrifuged and the supernatants were diluted to a final concentration of 0.8 - 1.5 g soluble protein/litre as described by Chamness et al. (1975). Larger amounts of normal human breast tissue

than of human or rat uterus were required for homogenization because of the lower yield of soluble protein obtained from breast tissue.

Protamine sulphate (Grade II, Sigma) was added to aliquots of diluted cytosol or serum in glass or plastic tubes (12 x 75 mm) pretreated as described by Chamness et al. (1975) to give a final concentration of 0.5 g/l. Vacant oestrogen binding sites in the precipitates (obtained by centrifugation at 2000 x g for 10 min) were then submitted to saturation analysis at 4°C; vacant and occupied oestrogen binding sites were measured by the oestradiol-17 β exchange assay at 30°C.

2.2.1 Saturation analysis for vacant binding sites

Duplicate tubes containing known quantities of the protamine precipitates from rat or human tissues and 10,20,30,40,60 or 80 pg of 2,4,6,7-(n)³H - oestradiol-17 β were incubated at 4°C overnight in a total volume of 0.5 ml. Parallel incubations of the protamine containing precipitates ^{with} the same concentrations of ³H - oestradiol-17 β plus 500 nmol/l non-radioactive oestradiol-17 β permitted determination of non-specific binding. The data was plotted according to Scatchard (1949). The concentration of vacant oestrogen binding sites and the dissociation constant of binding (K_d) were calculated from the intercept and gradient of the graph respectively.

2.2.2 Exchange assay for total binding sites: rat tissues

Triplicate tubes containing the protamine precipitates from cytosols or sera in Tris-dithiothreitol buffer (0.5 ml) containing ³H - oestradiol-17 β (10 nmol/l) were incubated overnight at 4°C and subsequently for 3h at 30°C, except where other concentrations or times were studied (as indicated in the text). Parallel incubations of the same composition but including oestradiol-17 β (500 nmol/l) were carried out to measure non-specific binding.

The number of occupied oestrogen binding sites that could be measured by exchange assay following artificial saturation of a known number of vacant binding sites was initially determined by saturation analysis at 4°C of an unsaturated rat cytosol. This cytosol was then artificially saturated by incubation with non-radioactive oestradiol-17 β (10 nmol/l) for 2h, followed by treatment with dextran-coated charcoal to remove excess free oestradiol-17 β . Exchange assay was then carried out for 1, 2, 3, 4 and 6h at 15°C, 22°C and 30°C by incubation with ³H-oestradiol-17 β (10 nmol/l) after overnight incubation at 4°C.

2.2.3 Exchange assay for total binding sites: human tissues

The assay conditions for human tissues were slightly modified from those used for rat tissues in that the overnight incubation at 4°C was omitted. The number of occupied oestrogen binding sites that could be measured by exchange assay in an artificially saturated human cytosol was also measured. The concentration of vacant binding sites was initially determined by saturation analysis of an unsaturated human uterine cytosol. This cytosol was then artificially saturated by incubation with non-radioactive oestradiol-17 β (15 nmol/l) for 2h followed by treatment with dextran-coated charcoal. Exchange assay was then carried out, either immediately by heating at 30°C for 1, 2, 3 or 4 h or with an overnight preincubation at 4°C followed by heating the next morning for 1, 2, 3 or 4 h.

Separation of free and bound hormone At the end of the incubation with ³H-oestradiol-17 β , the precipitates from saturation analysis and exchange assay were washed thrice with Tris buffer (2 ml) at 4°C. The radioactivity bound to washed precipitates was extracted twice with 1 ml portions of ethanol. Dried extracts were counted after dissolving in toluene (5 ml containing PPO 0.4% w/v) using a liquid scintillation counter (isocap β , Searle).

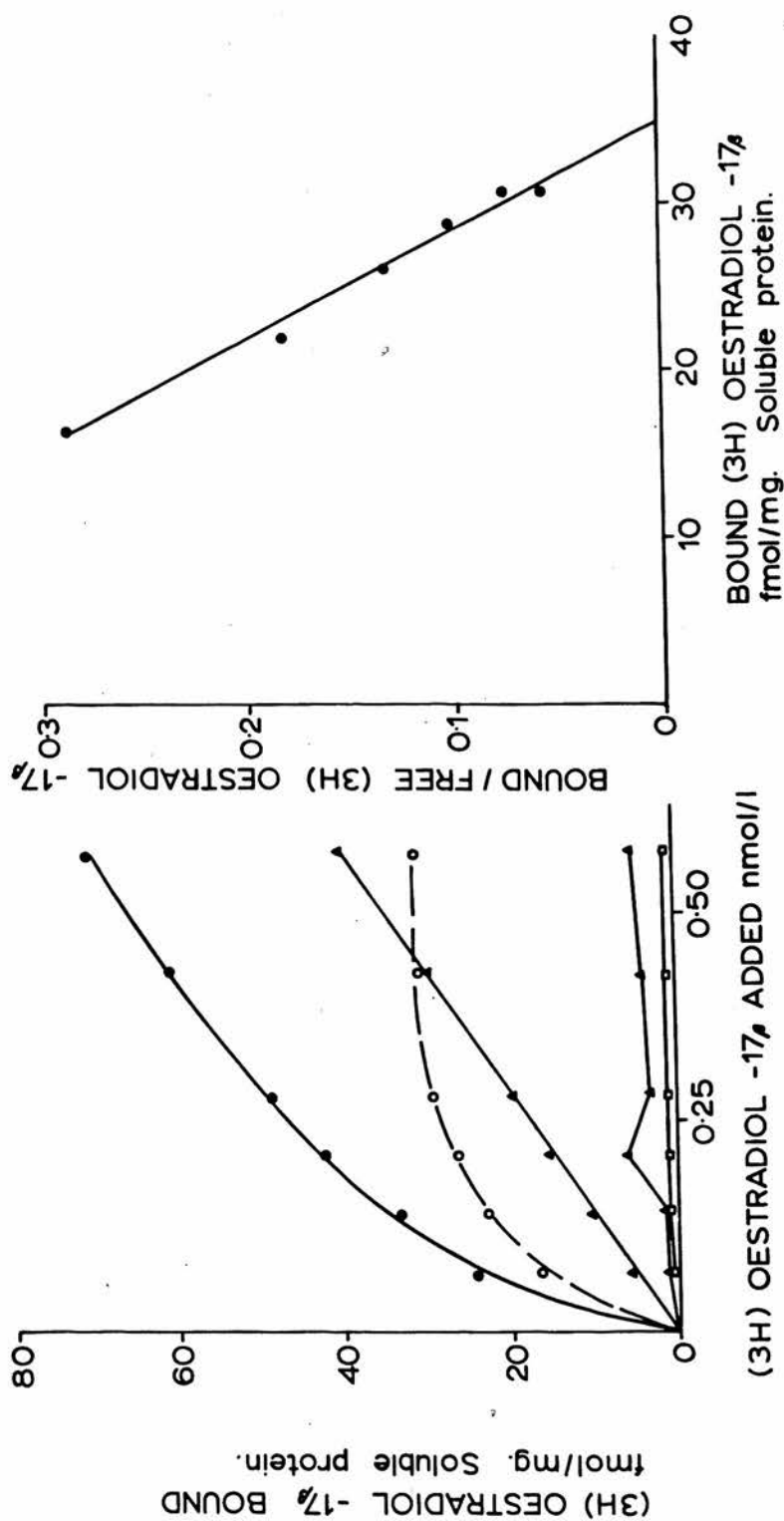


Fig. 2.1:

Determination of the number of vacant and specific binding sites for oestradiol-17 β in mammary cytosol from a lactating rat.

Left Protamine precipitates from cytosols were incubated overnight at 4°C in various concentrations of 3H-oestradiol-17 β (●) or 3H-oestradiol-17 β plus 500 nmol/l non-radioactive oestradiol-17 β (▲) and the binding of 3H-oestradiol-17 β determined; specific oestradiol-17 β binding (○) was obtained by subtraction of the latter from the former. Specific oestradiol-17 β binding of rat serum (▲) and rat muscle (□) are included for comparison.

Right Scatchard analysis of above data for mammary cytosol.

Tests of statistical significance The statistical significance of differences was examined by Student's t-test.

2.3 RESULTS

2.3.1 Rat Tissues

Saturation analysis High affinity binding of ^3H - oestradiol- 17β by vacant binding sites present in cytosol from rat mammary tissue (Fig. 2.1) and rat uterus (not shown) was evident upon Scatchard analysis. In contrast, muscle cytosol and rat serum (Fig 2.1) did not show any high affinity binding.

Effect of time, temperature and ^3H - oestradiol- 17β concentration on the binding of ^3H - oestradiol- 17β to protamine-precipitated rat uterine cytosol

At 4°C , linear Scatchard plots were obtained when the protamine precipitates from rat uterine cytosol was incubated with low concentrations of ^3H - oestradiol- 17β (not shown). However, if the protamine precipitate was incubated at 30°C for 3h instead of 4°C overnight, then lower binding of ^3H - oestradiol- 17β was seen for all concentrations of ^3H - oestradiol- 17β less than 2 nmol/l (Fig. 2.2). This was presumably due to the destruction of vacant receptor by heating at lower concentrations of ^3H - oestradiol- 17β . In the exchange assay 5 or 10 nmol/l ^3H - oestradiol- 17β was therefore used.

In 12 rat uterine cytosols an exchange assay was carried out for 3h at 30°C with either 5 or 10 nmol/l ^3H - oestradiol- 17β ; mean non-specific binding was 38 and 53% of the total bound ^3H - oestradiol- 17β respectively. It was found that incubation with 5 nmol/l ^3H - oestradiol- 17β gave only $95 \pm 16\%$ ($n=12$) of the receptor concentration which was detected when 10 nmol/l ^3H - oestradiol- 17β was used. 10 nmol/l ^3H - oestradiol- 17β was therefore used routinely in all subsequent assays.

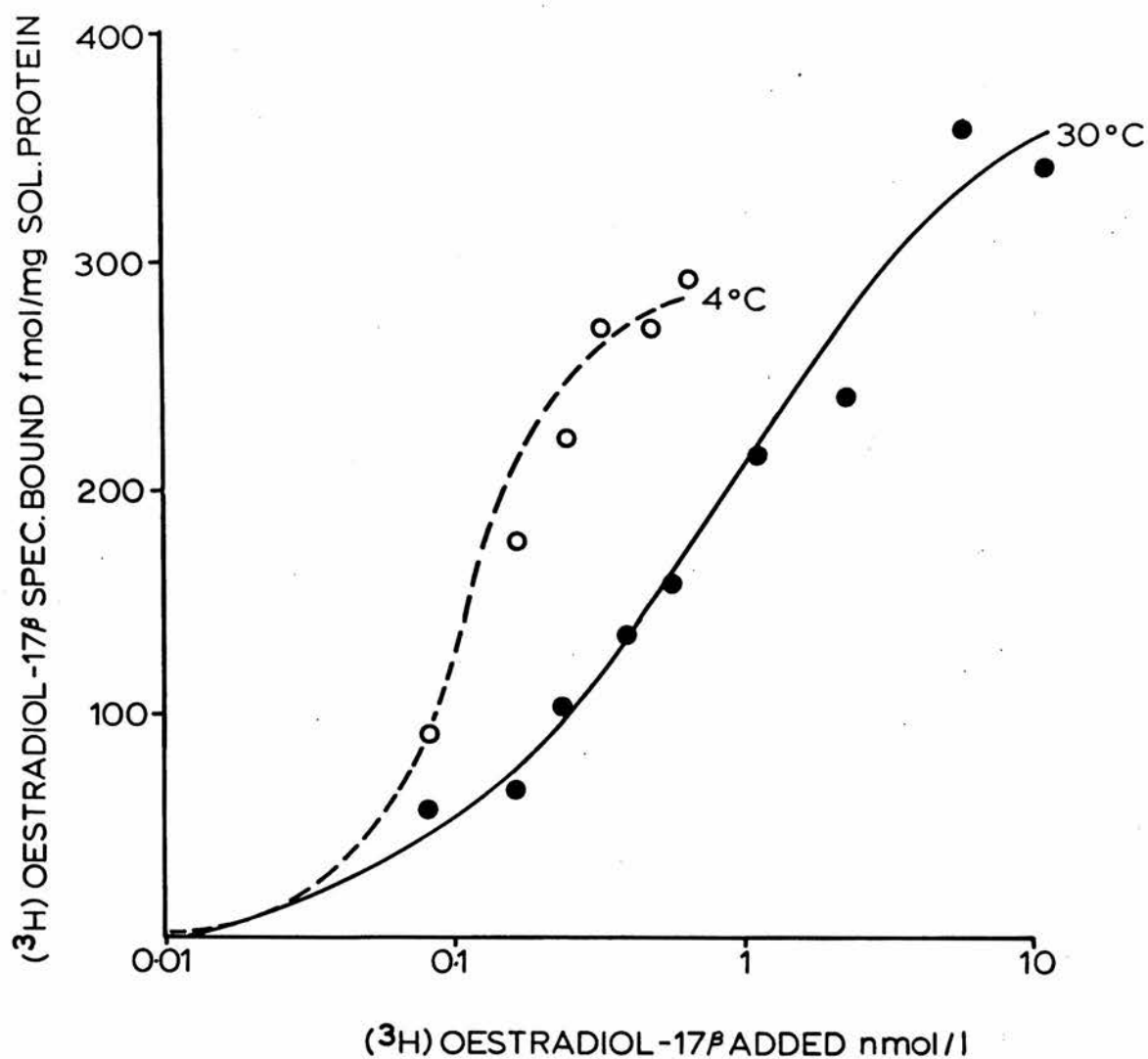


Fig. 2.2: The specific binding of ^3H -oestradiol- 17β to the protamine-precipitate from rat uterine cytosol following (o) non-exchange incubation at 4°C overnight, or (●) exchange incubation (at 4°C overnight, then at 30°C for 3h), for concentrations of ^3H -oestradiol- 17β from 0.08 nmol/l to 10 nmol/l. Non-specific binding was subtracted in all cases.

Incubation at higher temperature allowed the exchange of added ^3H -oestradiol- 17β for endogenous oestradiol- 17β which was already bound, as well as resulting in the production of bound ^3H -oestradiol- 17β from vacant binding sites. An attempt was made to determine the optimum temperature and incubation period for exchange and the fraction of bound sites that could be detected by saturating a cytosol with excess non-radioactive oestradiol- 17β followed by incubation with 10 nmol/l ^3H -oestradiol- 17β . It was found that optimum exchange of added ^3H -oestradiol- 17β for bound oestradiol- 17β occurred after 2 - 3 h at 30°C (Fig. 2.3). However, only 65% of the sites detected by saturation analysis before artificial saturation could be detected by exchange assay. Thus a correction factor of 100/65 was applied to all subsequent exchange assay results.

Changes in vacant and occupied uterine binding sites and total binding site concentration in rat muscle, serum and mammary cytosol during the oestrous cycle

The total binding site levels in uterine cytosol were measured in 18 rats at various stages of the oestrous cycle, and in six of the experimental animals vacant and occupied binding sites in muscle cytosol were also measured. The concentration of occupied binding sites was obtained by subtracting the vacant binding site value from total binding site value.

The mean concentration of vacant binding sites was significantly lower at pro-oestrus than at oestrus ($P < 0.001$), metoestrus ($P < 0.05$) or dioestrus ($P < 0.01$). No vacant sites were detected in either serum or muscle.

Mean total binding site levels were significantly higher at oestrus than at pro-oestrus ($P < 0.01$) or metoestrus ($P < 0.05$), and levels at dioestrus were also significantly higher than those at pro-oestrus ($P < 0.01$, Fig. 2.4). The variation in mean total and vacant

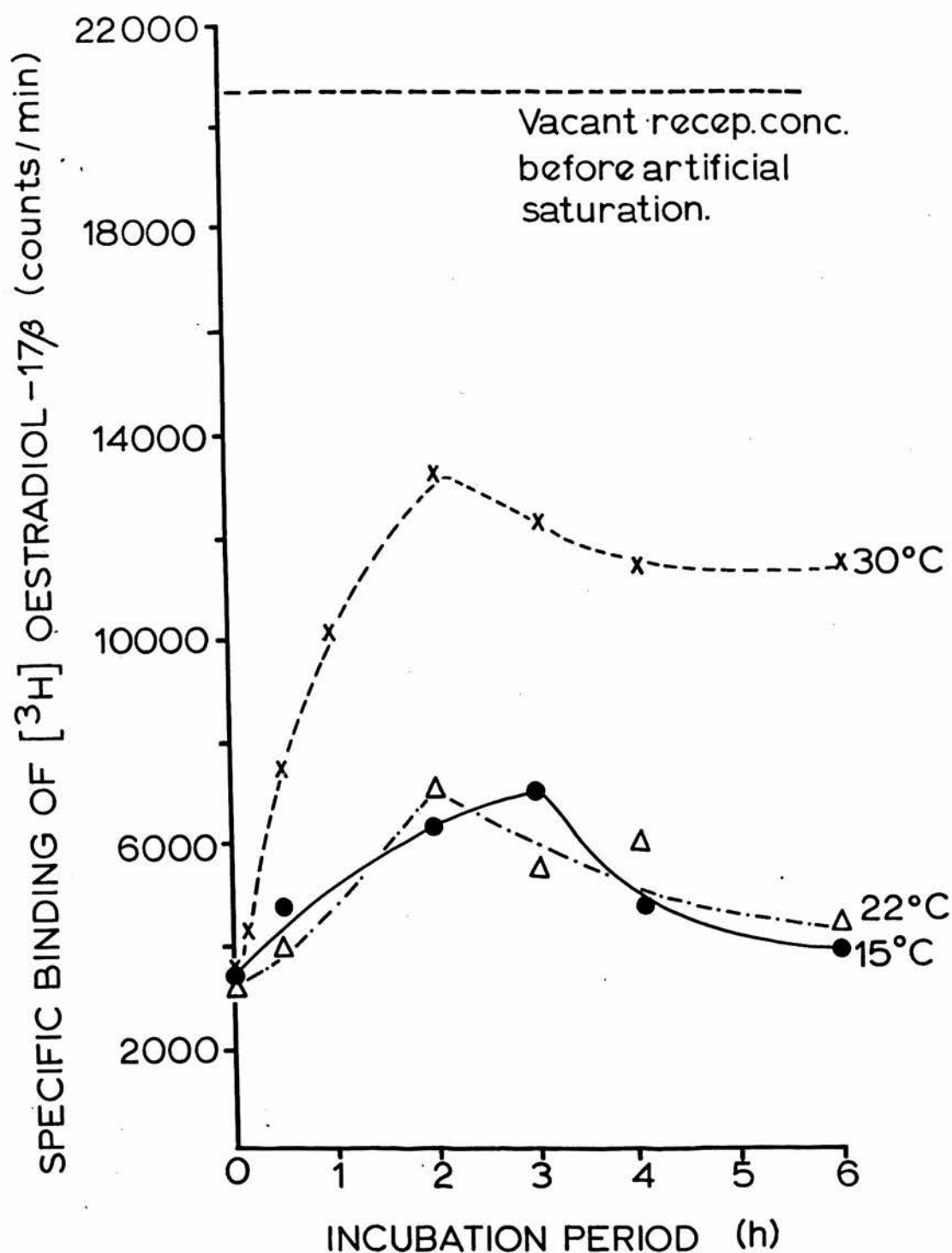


Fig. 2.3: The exchange of non-radioactive oestradiol- 17β and $3H$ -oestradiol- 17β in a rat uterine cytosol artificially saturated by incubation with 10 nmol/l non-radioactive oestradiol- 17β for 2 h followed by treatment with charcoal to remove excess, free non-radioactive oestradiol- 17β . After treatment with protamine sulphate the precipitates were incubated at (●) 15, (Δ) 22 and (x) 30°C in 10 nmol/l $3H$ -oestradiol- 17β for 1, 2, 3, 4 and 6h. The number of vacant sites before artificial saturation, determined by saturation analysis at 4°C, is shown by the broken line. Non-specific binding was subtracted in all cases.

binding site concentrations was inversely related to concentrations of plasma oestradiol-17 β which were highest at pro-oestrus and lowest at oestrus (Fig. 2.4). It was also observed that in 14 out of the 18 rat uterine cytosols (including all six of the cytosols from animals in oestrus) the concentration of vacant binding sites (determined by saturation analysis) exceeded the concentration of vacant binding sites (determined by exchange assay) before the use of the 100/65 correction factor described above.

It was found that the mean total binding sites for uterus at all stages of the oestrous cycle significantly exceeded the value obtained for muscle or serum ($P < 0.001$). During the oestrous cycle, the changes in either occupied binding sites or the degree of occupancy of binding sites did not attain statistical significance. This may have been caused by the wide variation in the degree of occupancy of binding sites observed in individual animals during the same phase of the oestrous cycle or by continual intra-nuclear translocation of receptor-bound oestradiol-17 β .

A low concentration of vacant binding sites was found in the inguinal mammary glands from five lactating rats, the mean level being significantly lower than the mean values found in rat uterine cytosol at all phases of the oestrous cycle ($P < 0.001$ in all cases). The mean total binding site concentration found by exchange assay was also significantly lower in rat mammary cytosol from lactating rats than the mean total uterine receptor concentration at all phases of the oestrous cycle ($P < 0.001$ in all cases) and significantly higher than the mean value for rat muscle ($P < 0.001$) and rat serum ($P < 0.001$). However, the value of bound sites was nearly eight times greater than the value for vacant sites (Fig. 2.4). The mean K_d value in the mammary cytosol from lactating rats was not significantly different from the mean K_d value seen during pro-oestrus but was significantly lower than the mean value found during oestrus ($P < 0.005$), metoestrus and dioestrus ($P < 0.01$ in both cases, Fig. 2.5).

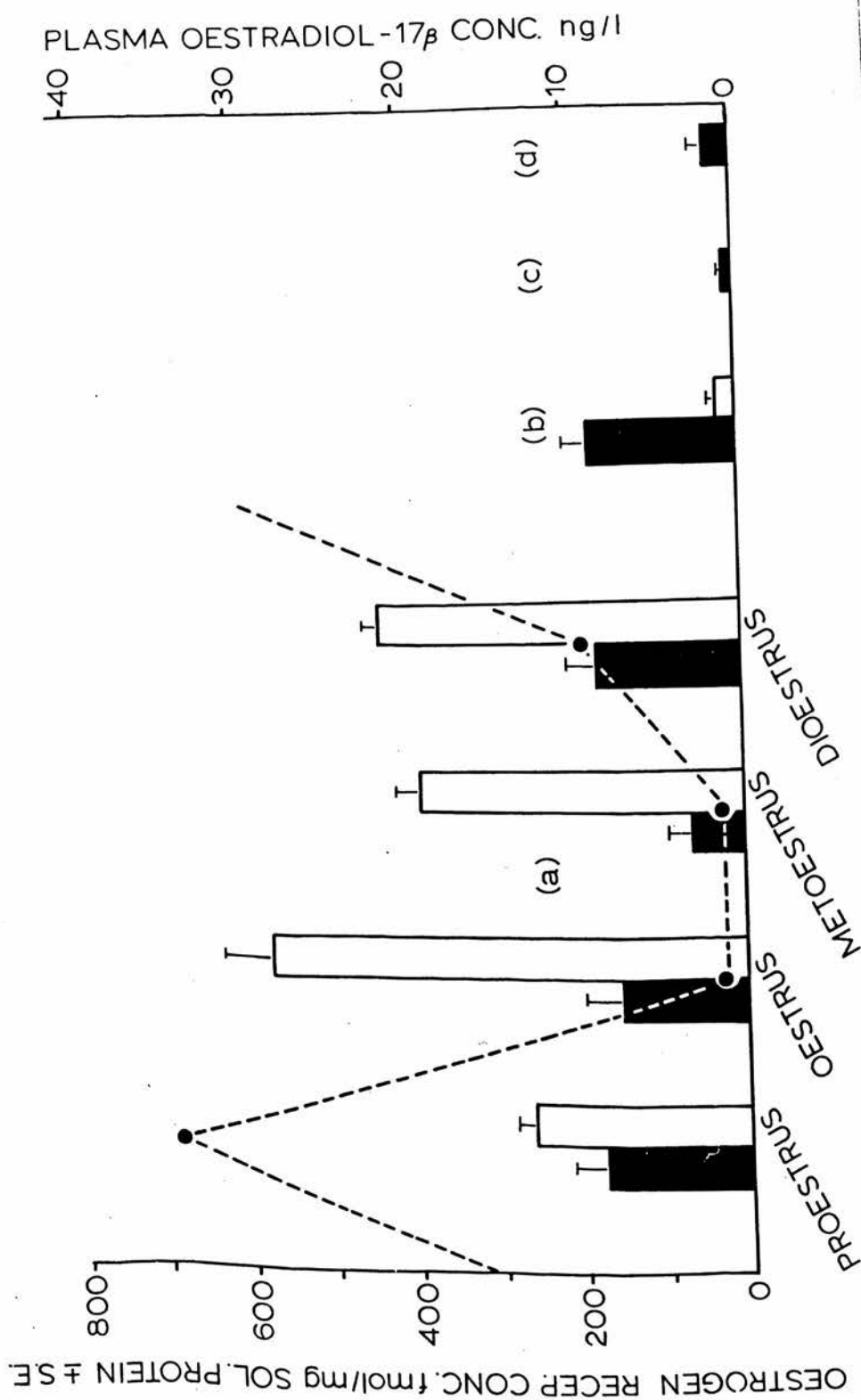


Fig. 2.4: The variation in vacut and occupied binding site concentrations in (a) uterine cytosol during the oestrous cycle in relation to plasma oestradiol-17 β concentrations (●) (proestrus, oestrus: n=6; metoestrus, dioestrus: n=3); (b) mammary cytosol from lactating rats (n=5); (c) muscle cytosol (n=6) and (d) diluted serum (n=6) in the rat. Mean occupied binding site concentrations \pm S.E.M. are indicated by the solid bars and mean vacut binding site concentrations \pm S.E.M. by the open bars. Non-specific binding was subtracted in all cases and was on average 53, 87, 96 and 86% of the total 3H-oestradiol-17 β bound for (a), (b), (c) and (d) respectively in exchange assay. Plasma oestradiol-17 β levels were measured only during the oestrous cycle and the S.E.M. (omitted from the figure for clarity) was 3.0, 0.4, 0.1 and 3.0 ng/l during proestrus, oestrus, metoestrus and dioestrus respectively.

2.3.2 Human Tissues

Saturation Analysis

Vacant binding sites could be detected in the cytosols from only uterus of all the human tissues studied. The mean value for the vacant binding site concentration in uterine cytosols was 141 fmol/mg soluble protein (Fig. 2.6). The dissociation constant (K_d) for uterine cytosols ranged from 0.26×10^{-10} to 5.62×10^{-10} . By contrast no vacant sites could be demonstrated in mammary cytosol, muscle cytosol or diluted serum (Fig. 2.6).

Exchange Assay

In an artificially-saturated human cytosol, it was found that after exchange reaction for 3h at 30°C, the number of total binding sites (measured by exchange assay) exceeded the number of vacant binding sites (measured by saturation analysis) present before artificial saturation. This suggested that exchange was also occurring with occupied oestrogen binding sites that were present before artificial saturation under these conditions (Fig. 2.7). Pre-incubation at 4°C overnight with 10 nmol/l ^3H -oestradiol-17 β before exchange assay as described above for rat tissues was omitted since this procedure resulted in a minor reduction (7-8%) in exchangeable binding sites (not shown). Pre-incubation at 4°C was therefore omitted from all subsequent exchange assays.

When mammary cytosol from a patient undergoing reduction mammo-plasty, muscle cytosol or diluted serum were incubated with 10 nmol/l ^3H -oestradiol-17 β for 1, 2, 3 or 6h, maximal binding was found in mammary cytosol after 3h at 30°C but neither muscle cytosol nor diluted serum showed a clear peak of binding (Fig. 2.7). Furthermore, the maximal levels of binding in mammary cytosol were higher than those found with muscle cytosol or diluted serum, suggesting that mammary cytosol, like uterine cytosol, contained occupied oestrogen binding sites.

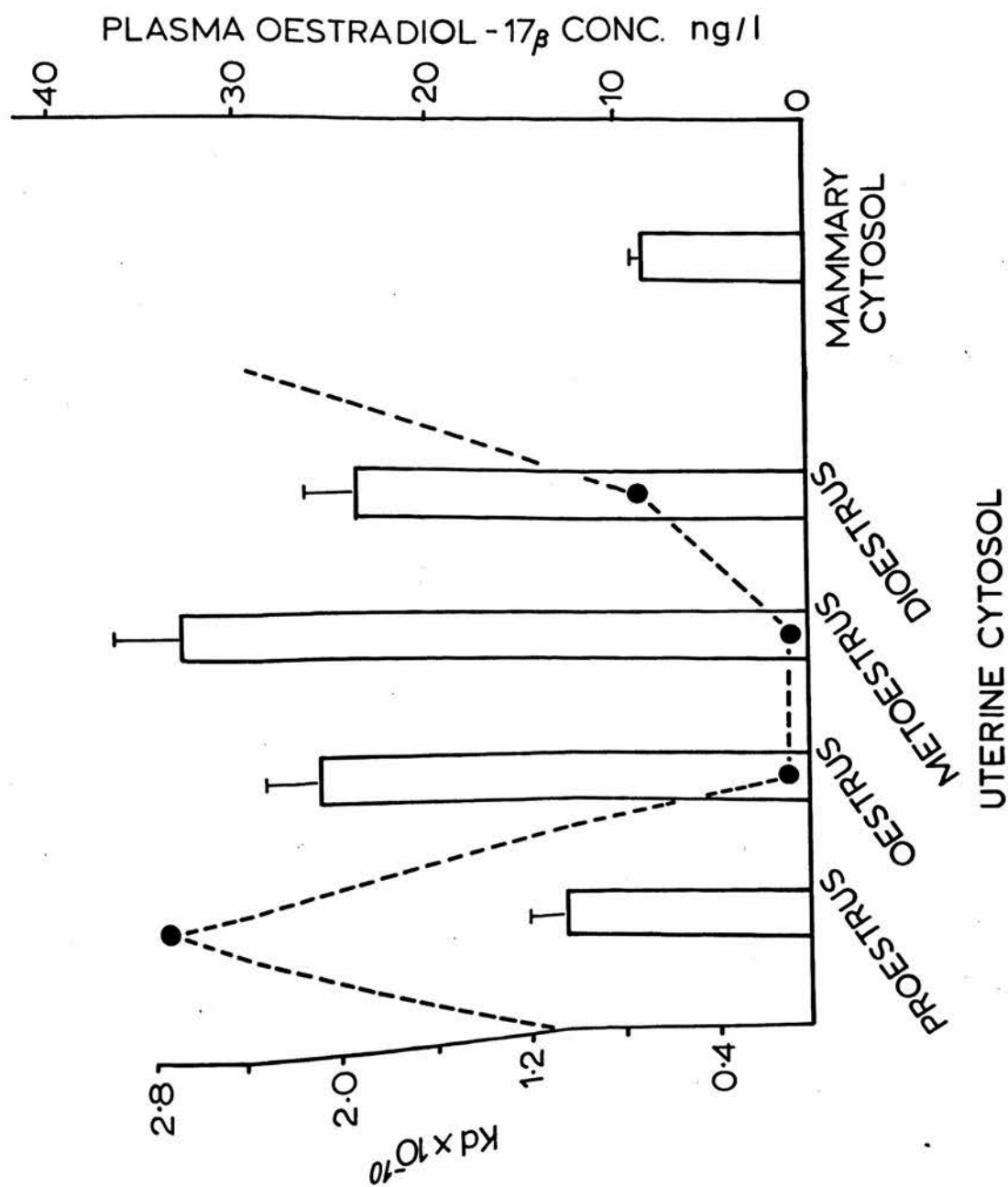


Fig. 2.5:
The variation in K_d in rat uterine cytosol during the oestrous cycle and in mammary cytosol from lactating rats using the same cytosols as described in Fig. 2.4. Plasma oestradiol- 17β levels (•) were determined during pro-oestrus, oestrus, metoestrus and dioestrus. The S.E.M. are the same as shown on Fig. 2.4.

In four human uterine cytosols studied using the exchange assay, total binding sites (vacant and occupied) exceeded the vacant site value, again suggesting a low concentration of occupied receptors in human uterine cytosol.

The mean value (\pm S.E.M.) of total binding sites in 14 mammary cytosols obtained for 13 pre-menopausal patients was 24 ± 3 fmol/mg soluble protein. This value significantly exceeded the mean values for muscle cytosol ($P < 0.01$, Student's t-test) and diluted serum ($P < 0.01$) but was eight-fold lower than the mean value of 164 ± 42 fmol/mg soluble protein for uterine cytosol (Fig. 2.6).

2.4 DISCUSSION

The purpose of the studies described in this chapter was to develop and validate a receptor assay that would permit detection of occupied cytoplasmic receptor sites in normal human breast tissue, as vacant receptor sites in previous studies (Feherty *et al.*, 1971; Leclercq *et al.*, 1975) were below the limit of detection. However, it was found that the exchange assay, based on protamine sulphate precipitation resulted in a detectable loss of cytoplasmic receptor sites in rat but not human uterus. Nuclear receptor sites were not measured as some doubt exists about their interpretation (e.g. Laing, Smith, Calman, Smith and Leake, 1977; Kiang, 1977); furthermore, the number of nuclear occupied receptors is partly dependant upon the level of available vacant cytoplasmic receptors (Sakai and Saez, 1976).

The concentration of ^3H -oestradiol- 17β used for exchange assay has varied from 5 nmol/l (Nicholson, Golder, Davies and Griffiths, 1976) to 30 nmol/l (Katzenellenbogen, Johnson and Carlson, 1973). A concentration of 10 nmol/l was chosen since apparent total receptor values were 5% lower, at 5 nmol/l. This may be due either to receptor breakdown since Katzenellenbogen *et al.* (1973) reported that high concentrations of

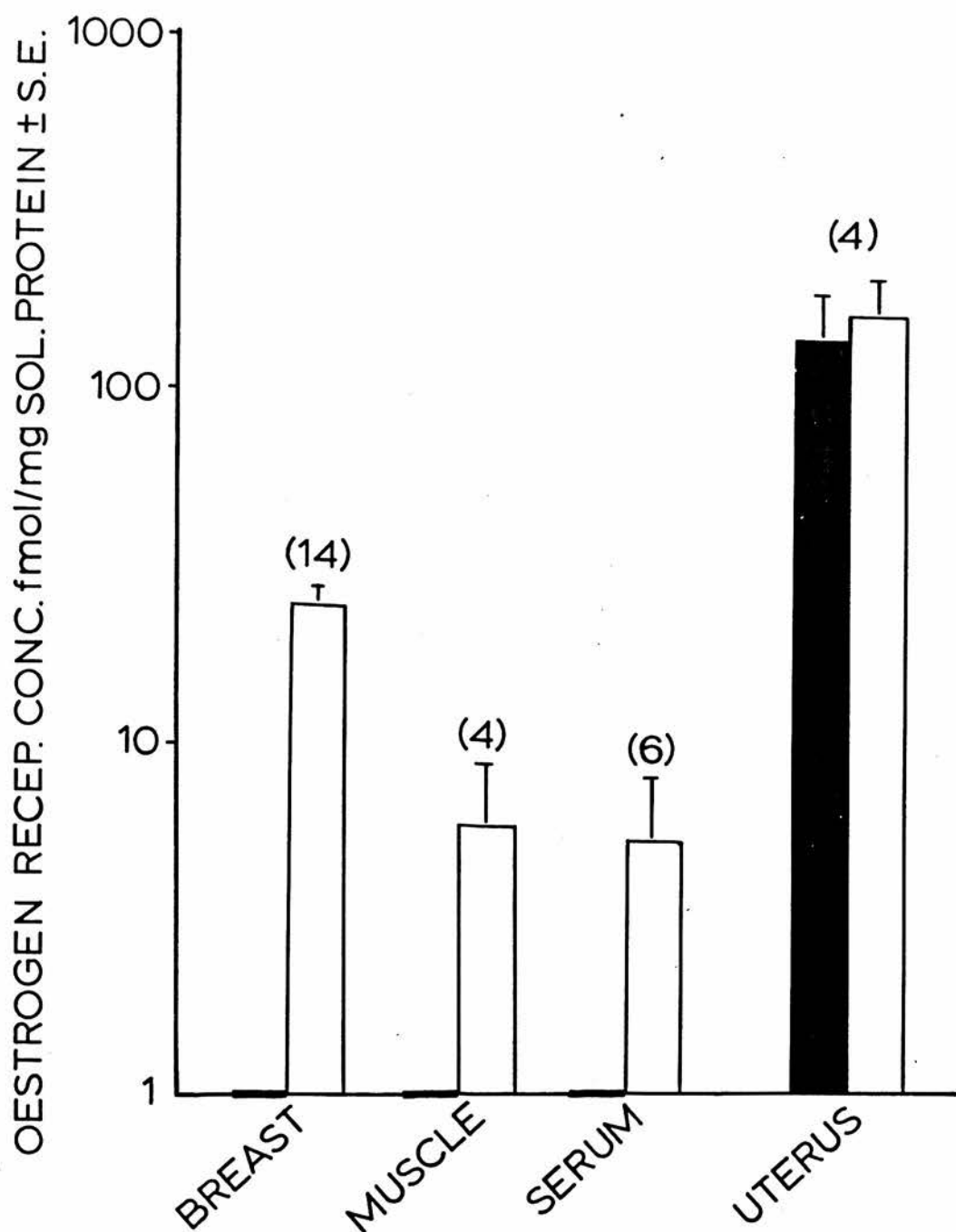


Fig. 2.6:

Vacant and total receptor concentrations in human breast cytosol, muscle cytosol, diluted serum and uterine cytosol. Mean vacant binding site concentrations \pm S.E.M. are indicated by the solid bar and mean total binding site concentration (vacant and occupied) \pm S.E.M. are indicated by the open bars. The number of experiments for each tissue is indicated in parentheses. Non-specific binding was subtracted in all cases in the exchange assay and was 81, 94, 91 and 79% on average of the total ^3H -oestradiol- 17β bound for breast cytosol, muscle cytosol, diluted serum and uterine cytosol respectively.

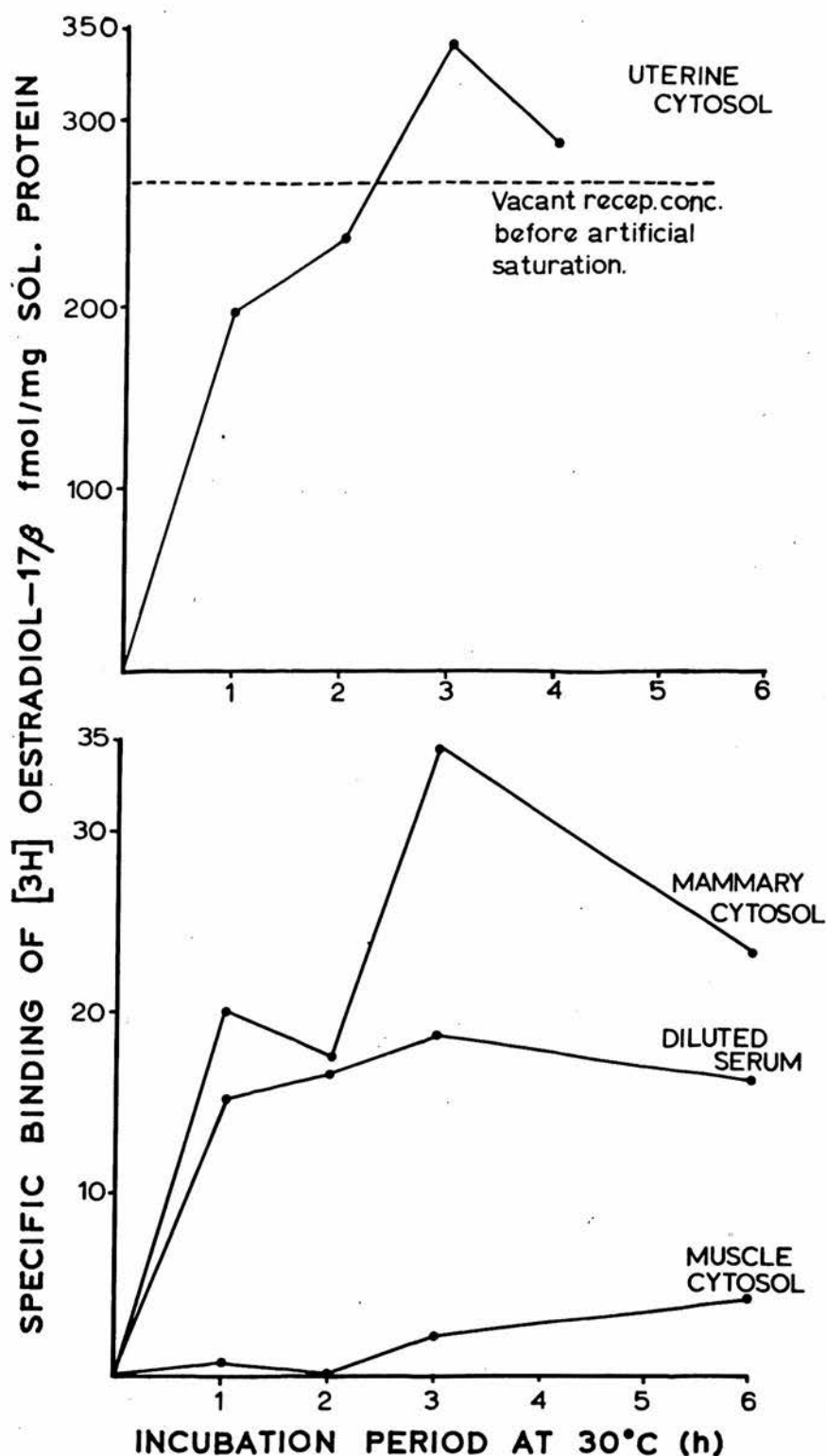


Fig. 2.7:

Above: The time course of exchange of non-radioactive oestradiol- 17β for $3H$ -oestradiol- 17β in an artificially saturated human uterine cytosol. A human uterine cytosol was incubated with 15 nmol/l non-radioactive oestradiol- 17β for 2h followed by treatment with dextran-coated charcoal to remove excess, free, non-radioactive oestradiol- 17β . Exchange assay was then carried out for 1, 2, 3, or 4h at $30^{\circ}C$. Saturation analysis was also performed on the uterine cytosol at $4^{\circ}C$ before artificial saturation to yield the concentration of vacant receptor sites before artificial saturation. Non-specific binding was subtracted in all cases.

Below: the time course of binding of $3H$ -oestradiol- 17β in human mammary cytosol, diluted serum and muscle cytosol when exchange assay was carried out by incubation at $30^{\circ}C$ for 1, 2, 3 and 6h. Non-specific binding was subtracted in all cases.

^3H -oestradiol- 17β are necessary to stabilize the receptor, or to the greater dilution of ^3H -oestradiol- 17β by endogenous hormone. The use of 30 nmol/l ^3H -oestradiol- 17β in assays involving protamine-precipitated receptors would, however, increase the non-specific binding to unacceptably high levels and thereby diminish sensitivity.

During the oestrous cycle, vacant binding site concentrations varied considerably, being minimal in pro-oestrus and maximal in oestrus in agreement with previous observations (Feherty, Robertson, Waynforth and Kellie, 1970; Lee and Jacobson, 1971; Shain and Barnea, 1971) and in inverse relationship to the plasma oestradiol- 17β concentration. Similar fluctuations also occur in rat mammary tumours (Hawkins, Hill, Freedman, Killen, Buchan, Miller and Forrest, 1977). However, in the study of Hawkins *et al.*, (1977) using an assay not involving protamine sulphate, maximal receptor concentrations were detected in metoestrus.

In the studies described in this chapter the concentrations of occupied binding sites varied to a lesser extent than did the concentrations of vacant binding sites but the total binding site concentrations were also significantly increased at oestrus. This may reflect increased receptor synthesis induced by the oestrogen secreted in pro-oestrus since several studies have shown that injection of exogenous oestradiol- 17β initially depletes, but subsequently increases, the cytoplasmic receptor concentration (Sarff and Gorski, 1971; Nicholson *et al.*, 1976; Pavlik and Coulson, 1976). The changes in concentration of occupied sites were not statistically significant, possibly because, once formed, receptor-bound oestradiol- 17β is rapidly translocated out of the cytoplasm into the nucleus to maintain an equilibrium between the two subcellular compartments (Williams and Gorski, 1971).

A low but detectable concentration of vacant oestrogen binding sites was found in the inguinal mammary glands of 21 day lactating rats, a result in accord with earlier observations (Shyamala and Nandi, 1972; Witliff *et al.*, 1972). However, an apparently eight-fold greater concentration of occupied sites was demonstrated by exchange assay, an unexpected result in view of the fact that oestrogen concentrations in post-partum lactating rats are very low (R.A. Hawkins and E. Killen, personal communication). One possible explanation for this finding is that a steroid-binding component might be present in rat milk.

Using the exchange assay, low levels of ^3H -oestradiol-17 β were bound by rat skeletal muscle cytosol (8 ± 12 fmol/mg soluble protein) and rat serum (33 ± 3 fmol/mg soluble protein) but no vacant binding sites were detected by saturation analysis. Although oestrogen receptors are very widely distributed in animal tissues (e.g. Brinkmann, Mulder, Lamers-Stahlhofen, Mechielsen and van der Molen, 1972) and this binding in muscle might represent occupied sites, it would be preferable to regard the level observed in muscle as the limit of sensitivity of the exchange method. The binding seen with serum, presumably caused by precipitated serum proteins, will also need to be taken into account in tissues where serum contamination is significant.

During the oestrous cycle, the apparent K_d for the binding of the vacant receptors changed significantly, being lower in pro-oestrus than in metoestrus or dioestrus. A similar result was found by Wise, Payne, Karsch and Jaffe (1975) who showed a reduction in K_d in the pituitary gland after the administration of oestrogen to sheep. Hawkins *et al.* (1977) however found the contrary effect in rat mammary tumours, where the apparent K_d was probably less accurately determined when receptor concentrations were low. The reasons for these changes in apparent K_d and their significance are unclear.

Among the human tissues studied, a low but detectable level of occupied oestrogen binding sites was found in normal human breast tissue. The mean level is approximately eight times lower than the total oestrogen binding site concentration seen in the human uterus, a finding which suggests that there may be few oestrogen-sensitive cells present in the normal breast. Alternatively, if it is assumed that oestrogen receptors are found in stroma, ductal and glandular tissue, then the number of oestrogen receptor sites in each cell must be minute. It was not possible to distinguish between the two alternatives because of the difficulty in obtaining biopsy samples that consisted of only one type of tissue. However, oestrogen receptors have been detected in approximately 11-50% of benign breast dysplasias and fibroadenomas (Leclercq *et al.*, 1975; Rosen, Menendez-Botet, Nisselbaum, Urban, Mike, Fracchia and Schwartz, 1975) where the epithelial component of the tissue has a higher cellular density than that found in normal breast tissue. This suggests that the low concentrations of oestrogen binding sites seen in normal breast tissue might be due to the scarcity of epithelial cells. It is likely that the normal breast also contains a low level of vacant binding sites but these may be below the limit of detection of the assay method, due to vacant receptor lability.

The loss of receptor sites that was found on exchange assay is probably due to the increased thermolability of free receptor in comparison with bound receptor (Katzenellenbogen *et al.*, 1973). Thus incubation of cytosol at temperatures which permit exchange to occur is likely to result in destruction of a proportion of both vacant receptors and receptors which become vacant transiently by the dissociation of endogenous oestradiol from occupied receptors. A loss of 35% of oestrogen binding sites was observed in an exchange assay performed on a rat uterine cytosol that was artificially saturated by previous incubation with non-radioactive oestradiol- 17β , and a correction factor of 100/65 was

therefore used to compensate for this loss. This loss of oestrogen binding sites was also observed in 14 out of 18 rat uterine cytosols examined where, in the absence of the application of such a correction factor, the value for vacant binding sites (determined by saturation analysis) was greater than the value for total binding sites (determined by exchange assay). In particular, for all the cytosols from animals in oestrus, (where the largest number of vacant receptors was detected), greater binding values were obtained by saturation analysis than exchange assay. It should be noted that it was not possible to estimate occupied receptor sites before artificial saturation, as exchange assay would have resulted in the receptor losses that this experiment was attempting to estimate, and that no independent method for checking occupied site concentration appears to be present in the literature concerning exchange assays.

The receptor losses observed are similar to those of Nicholson et al. (1976) who reported a 25% loss of oestrogen binding sites, and to those of Richards (1975) who also reported loss of cytoplasmic oestrogen receptor at temperatures greater than 4°C; in both cases an exchange assay that did not utilise protamine sulphate was used. However, the originators of the method used, Chamness et al., (1975) did not report any such loss. The reason for this apparent discrepancy is not clear but it may be related to the difference in the method of detection of vacant sites before artificial saturation, as Chamness et al. (1975) used an equilibrium time of only three hours, in contrast to the 18 hours they originally reported (McGuire and de la Garza, 1973). In a more recent modification of the method involving protamine sulphate by Lippman and Huff (1976), an intermediate recovery (86%) of receptor sites was found after artificial saturation of a human breast tumour cytosol.

In an experiment using a human uterine cytosol that had been artificially saturated, no such loss of binding sites in the exchange

assay was observed. This may have been due to the presence of greater numbers of occupied receptors before artificial saturation or it may be a characteristic of the different types of tissue studied. Separate correction factors were therefore required for rat and human tissues and exchange assay results should perhaps be regarded as indicating qualitative rather than quantitative changes.

It is possible that errors in vacant site quantitation may have occurred due to the use of only a single high concentration of non-radioactive oestradiol-17 β (500 nmol/l) to determine non-specific binding, as it has been suggested that errors occur in Scatchard plots based upon the use of excessive amounts of non-radioactive oestradiol for the determination of non-specific binding (Chamness and McGuire, 1976). However, during the routine assay of a large number of human breast cancers by a dextran-coated charcoal method, it was found that the use of a 100 fold excess of non-radioactive oestradiol-17 β (over ^3H - oestradiol-17 β) lead to an overcorrection and consequent loss of linearity of the Scatchard plot compared with the use of a 2000 fold excess of non-radioactive oestradiol for the determination of non-specific binding (R.A. Hawkins, personal communication). It has also been suggested that a variation in specific activity of ^3H -oestradiol-17 β might affect the determination of occupied sites (Chamness and McGuire, 1976). However, in experiments on rat uterine cytosol, a change in specific activity by a factor of 20 does not alter the slope and intercept of the Scatchard plot (R.A. Hawkins, personal communication). Furthermore, the K_d values that were found for both rat and human uterine cytosol were of the same order of magnitude as published values (Hawkins, Hill and Freedman, 1975; Leclercq et al., 1975; Nicholson et al., 1976). It was therefore felt that the use of a single concentration of non-radioactive oestradiol did not affect vacant site quantitation.

In conclusion, three major difficulties were encountered in studies on human tissues. Firstly, high mean levels of non-specific binding (79 - 94%) were encountered in all the tissues studied. This undoubtedly affects the reproducibility of the method. Secondly, ^{'specific'} binding of ^3H -oestradiol- 17β was observed under exchange assay conditions in muscle and serum. The binding level in human muscle, like in rat muscle, could be regarded as the limit of the sensitivity of the exchange method. The binding level in serum, like in the rat, will need to be considered in tissues where serum contamination is significant. Thirdly, it was difficult to obtain reproducible samples of glandular tissue. In a previous study by Short and Drife (1977), wide histological variations were found within a single breast, with the proportion of glandular tissue varying from 0.3% to 29% of the area of the section; variations between individuals were very much greater than those within an individual.

For these reasons, it was felt that the exchange assay based upon protamine sulphate precipitation, while demonstrating that occupied cytoplasmic oestrogen binding sites were present in normal human breast, was not likely to yield useful information about the changes of the breast in response to the menstrual cycle, nor in response to a full term pregnancy. No further experiments were therefore undertaken using this assay.

CHAPTER 3

RADIOIODINATION OF IMMUNOGLOBULINS

3.1 INTRODUCTION

Radioimmunoassay (RIA) was chosen for the majority of immunoglobulin measurements described in this thesis because of the high degree of sensitivity and specificity associated with the method, and the requirement of only small volumes of material for assay. This chapter describes the iodination of immunoglobulins for RIA.

In any RIA, three reagents are required:

- (a) a purified preparation of the substance to be measured, for labelling with the appropriate radioisotope;
- (b) a preparation containing known amounts of the substance to be measured to act as standard; and
- (c) a specific antiserum with antibody activity against the substance to be measured.

It should be noted that in this and subsequent chapters, the immunoglobulins measured are acting as antigen, and not as antibody in RIA.

The aim of any radiolabelling procedure is to produce a stable, radiolabelled antigen (tracer) that is immunologically identical to the unlabelled substance to be measured. Among the radioisotopes available for labelling, γ -emitters are preferable to β -emitters due to the higher specific activity of γ -emitters. Radioiodine is particularly suitable as it is a γ -emitter that can be readily substituted into the tyrosine residues of proteins or polypeptides; ^{125}I is usually chosen because of its long half-life (60 days) and higher isotopic abundance (95%) in commercially available preparations, in preference to ^{131}I with its shorter half-life (8 days) and lower isotopic abundance (20%).

The preparation of the substance to be radiolabelled should be as pure as possible as this can lead to problems of specificity in RIA. For example, if 11S IgA is to be radiolabelled, it should not be contaminated with 7S IgA, IgG or IgM. Furthermore, no alteration of the substance to be radiolabelled should have occurred during purification, as occasionally happens during the extraction and purification of pituitary hormones.

Three methods are commonly used for the radiolabelling of proteins: the method of Hunter and Greenwood (1962) which involves oxidising ^{125}I using Chloramine T, with subsequent incorporation of ^{125}I into tyrosyl groups of the protein; the method of Marchalonis (1969) using the enzyme, lactoperoxidase; and the conjugation method of Bolton and Hunter (1972) which utilizes N-succinimidyl-3-(4-hydroxy, 5- ^{125}I -iodophenyl) propionate. Of the three methods, that of Hunter and Greenwood (1962) using Chloramine T results in the best yield of tracer and is technically the simplest, and was therefore chosen. During the radiolabelling procedure, conditions are optimised to ensure that the specific activity of the tracer (defined as the number of ^{125}I atoms incorporated per molecule of protein) is not excessive, as damage to protein molecules becomes increasingly worse with increasing specific activity (Hunter, 1973). It is now widely accepted that the incorporation of one iodine atom per molecule of protein is the most desirable specific activity, and this was confirmed at each iodination.

A loss of affinity of antiserum for tracer results in a loss of RIA sensitivity (Ekins, 1975). The tracer should therefore be assessed for the degree of immunological identity with the native, unlabelled protein and this can be achieved in three different ways: by physico-chemical methods e.g. gel filtration; by the binding of tracer to excess antibody; and by a direct comparison in RIA of the

immunoreactivity of the labelled and unlabelled material. These methods were used to assess the ^{125}I -immunoglobulin that was prepared for use in RIA.

3.2 MATERIALS AND METHODS

The following were used:

Reagents and Buffers

Sodium phosphate buffer (Na_2PO_4 ; 0.05M, pH 7.5 at room temperature) containing sodium azide (NaN_3 ; 0.01% w/v)

Na_2PO_4 (0.05M or 0.25M, pH 7.5), containing no NaN_3 .

Chloramine T; Sodium metabisulphite ($\text{Na}_2\text{S}_2\text{O}_5$); Potassium iodide (KI).

Crystallised Bovine Plasma Albumin (CBP; Armour Pharmaceuticals).

Na^{125}I (Amersham) as the carrier-free solution in NaOH, specific activity 100 mCi/ml.

Sephadex G50, Sepharose 4B (Pharmacia).

Sera and Antisera

Specific rabbit antisera (for antigenic specificities and sources, see Table 3-3).

Donkey anti-rabbit IgG serum (DARS; Wellcome or Scottish Antibody Production Unit).

Non-Immune Rabbit serum (NIRS; Wellcome).

Horse serum (HS; heat inactivated; Wellcome).

Immunoglobulins

The following purified Immunoglobulins were iodinated:

Secretory IgA (11S IgA), purified by the method of Newcomb,

Normansell and Stanworth (1968) and given by Mr. R.R. Samson.

Serum IgA (7S IgA), purified by ZnSO_4 and $(\text{NH}_4)_2\text{SO}_4$ precipitation and Sephadex G200 gel filtration as described by Schuurman (1977), and given by Dr. H.J. Schuurman.

Serum IgG, purified by polyethylene glycol precipitation and

DEAE ion exchange chromatography, and given by Dr. S. Moore.

All Immunoglobulins were stored in 63 x 11 mm stoppered polystyrene tubes at -20°C . 11S IgA and IgG were stored in 80 ug amounts at a concentration of 2mg/ml and 7S IgA was stored in 50 ug amounts at a concentration of 2.5 mg/ml.

Radioiodination

The following method was used for all iodinations:

To 300-450 uCi of $\text{Na } ^{125}\text{I}$ (for 11S IgA)

or

500-600 uCi of $\text{Na } ^{125}\text{I}$ (for 7S IgA or IgG)

in 75 x 12 mm polystyrene tubes, the following additions were made sequentially at room temperature:

1. 20 ul Na_2PO_4 (0.25 M, no NaN_3)
2. 20 ul Chloramine T (2.5 mg/ml in 0.05 M Na_2PO_4 , no NaN_3)
3. 20 ul 11S IgA (containing 40 ug 11S IgA)

or

10 ul 7S IgA (containing 25 ug 7S IgA)

or

20 ul IgG (containing 40 ug IgG).

The mixture was allowed to react for 10 seconds, then

4. 900 ul $\text{Na}_2\text{S}_2\text{O}_5$ (0.133 mg/ml in 0.05 M Na_2PO_4 , no NaN_3) was added to stop the reaction, followed by
5. 50 ul HS (to reduce non-specific binding).

The reaction mixture was mixed immediately after the addition of $\text{Na}_2\text{S}_2\text{O}_5$ and HS, and immediately transferred to a Sephadex G50 column (11S IgA, 7S IgA, IgG) and then to a Sepharose 4B column (11S IgA, 7S IgA) for additional purification of tracer.

Gel filtration

The eluting buffer in all gel filtration studies was Na_2PO_4 (0.05M containing 2% HS).

(a) Sephadex G50: 1 g. of Sephadex G50 was equilibrated overnight in an excess of Na_2PO_4 (0.05 M containing NaN_3 and 2% HS). The swollen gel was then decanted into a glass column (250 x 10 mm) containing a fixed porous plate. The column was then washed with at least 50 ml of Na_2PO_4 (containing 2% HS) and then run nearly dry once the gel was compacted. Immediately prior to use, 20 mg CBP in a volume of 1 ml buffer was run through the column to coat the Sephadex, and thus minimise adsorption of iodinated immunoglobulin to the column. Excess CBP was washed out of the column with a further 20-30 ml Na_2PO_4 . Each Sephadex G50 column was only used once for the purification of iodinated immunoglobulins.

Following Chloramine T radioiodination, the reaction mixture was applied to the top of the Sephadex column and allowed to percolate into the gel. The column was then topped up with Na_2PO_4 and 20 fractions of 1 ml volume were collected in 75 x 12 mm polystyrene tubes; each fraction was counted on a γ - counter.

(b) Sepharose 4B: A degassed preparation of Sepharose 4B was decanted into a 900 x 22 mm glass column containing a fixed porous plate (Wright Scientific Ltd.) until the column was completely filled. The gel was then washed thoroughly with at least 1 l Na_2PO_4 buffer prior to use. Following Sephadex G50 gel filtration, the fraction containing the highest radioactivity from the iodinated protein peak was placed on the top of the Sepharose 4B column, and allowed to percolate completely into the gel. The column was then topped up with Na_2PO_4 and 120 fractions of 3 ml volume were collected at room

temperature in 100 x 13 mm polystyrene tubes; each fraction was counted on a γ -counter. The column was then washed successively with 1 l of Na_2PO_4 containing 2% HS followed by 1 l of Na_2PO_4 with NaN_3 and then stored till the next iodination.

Confirmation of Immunological Identity of Tracer with unlabelled Immunoglobulin

(a) Gel filtration

The elution profile of radioactivity for each preparation of iodinated immunoglobulin was determined by gel filtration on Sepharose 4B.

(b) Binding of tracer to excess antibody

A pool was made from the fractions comprising each peak of radioactivity seen in the elution profile of iodinated 11S IgA on Sepharose 4B gel filtration.

A portion of each pool was tested with rabbit antisera each with different antigenic specificities at a range of final dilutions from $1/5000$ to $1/2,560,000$ using a six hour incubation period at room temperature. Antibody bound counts were then determined using a second antibody (DARS) and a carrier (NIRS) as described for routine immunoglobulin RIA (Chapter 4). The antigenic specificity of each rabbit antiserum used is described in Table 3-3; Peak I (Fig. 3.2) material was only tested with anti-7S IgA due to the limited amount of material available.

(c) Direct measurement of labelled and unlabelled immunoglobulins by RIA

Purified ^{125}I -11S IgA, ^{125}I -7S IgA and ^{125}I -IgG obtained by gel filtration using Sephadex G50 (all immunoglobulins) and Sepharose 4B (^{125}I -11S IgA, ^{125}I -7S IgA) were assayed directly by RIA by setting

up a standard curve containing known amounts of ^{125}I -immunoglobulin. A similar set of tubes were prepared containing known amounts of unlabelled immunoglobulin. A fixed mass of labelled immunoglobulin was added to all the tubes (as in the routine RIA, Chapter 4) followed by the specific antiserum, and the mixture incubated for six hours at room temperature. At the end of the incubation period, antibody bound counts were determined using a second antibody (DARS), and a carrier (NIRS), as described for routine RIA. The percentage of the original amount of iodinated immunoglobulin in each tube which was bound to antiserum was calculated, and plotted as a percentage bound, against the mass of immunoglobulin. The resultant curves for labelled and unlabelled immunoglobulin were then assessed by seeing if the two curves were superimposable (Fig. 3.4).

3.3 RESULTS

Calculation of percentage yield and specific activity of radioiodinated immunoglobulin

To enable a calculation of the specific activity of a radioiodinated immunoglobulin to be made, the following three factors must be known:

- (a) the weight of immunoglobulin used.
- (b) the amount of radioactivity used.
- (c) the proportion of (b) that is incorporated into (a) as a result of radioiodination.

A calculation of the specific activity and concentration of a preparation of iodinated immunoglobulin is required for two reasons. Firstly, the degree of iodination of the immunoglobulin preparation has a direct bearing on the quality of the tracer. Secondly, the RIA in this thesis depends upon the addition of a specific quantity of labelled



Table 3-1. The calculation of the percentage yield and specific activity of ^{125}I -11S IgA following Chloramine T radioiodination

40 ug 11S IgA iodinated with ^{125}I

<u>Elution from Sephadex G50</u>	
<u>Fraction no.</u>	<u>Counts/10s</u>
1	25
2	26
3	100
4	4521
5	23000*
6	11145
7	3284
8	1972
9	2366
10	2868
11	4603
12	7349
13	6035
14	2664
15	1118
16	646
17	453
18	232
19	160
20	25
<hr/>	
Total	81074

*fraction kept for use on Sepharose 4B column

Total radioactivity added = 83046cts/10S (equivalent to 377 uCi)

Total counts associated with protein (11S IgA)

= total counts added - eluted free ^{125}I

= 83046 - 28529

= 54517

\therefore % yield = $\frac{54517}{83046} \times 100 = 65.6\%$

\therefore Specific activity = 0.656×377

40

= 6.2 uCi/ug**

Of 54517 cts associated with 11S IgA, 23000 are in fraction 5

\therefore fraction 5 contains $\frac{23000}{54517} \times 40$

= 16.87 ug

Total radioactivity added = 83046 cts

Radioactivity remaining in reaction vessel = 8523cts

Radioactivity eluted from column = 81074cts

\therefore Radioactivity remaining on column = 83046 - (8523 + 81074) = 1972.

** one ^{125}I atom per molecule of 11S IgA is represented by a specific activity of 5.5 uCi/ug.

immunoglobulin, rather than the addition of a specific amount of radioactivity (in the form of labelled immunoglobulin).

Specific activity and concentration of tracer were determined by the following method (based on Greenwood, Hunter and Glover, 1963), and is illustrated with an example in Table 3-1.

(a) The total ^{125}I used for radioiodination is assessed just prior to the addition of the various reactants to the reaction vessel.

(b) The radioactivity associated with protein (immunoglobulin) is calculated as being that in (a) minus the counts eluted as 'free ^{125}I ' (see Fig. 3.1).

This figure is then used to calculate the % yield of ^{125}I -labelled immunoglobulin and hence the notional specific activity (Table 3-1).

(c) The counts in each fraction containing ^{125}I -labelled immunoglobulin (e.g. fractions 3-8, Table 3-1) are then expressed as a percentage of (b), and it is assumed that this percentage of the total immunoglobulin used (40 ug) is present in the respective fractions (e.g. see calculation for fraction 5, Table 3-1).

This method assumes that all the radioactivity that is not eluted as free ^{125}I is immunoglobulin associated, and may result in an overestimation. However, the error is likely to be small, as free ^{125}I (as iodide) does not readily adsorb to the reaction tube or the Sephadex column (Greenwood *et al.*, 1963) and is therefore readily eluted. Binding of iodinated protein is minimised by prior adsorption of the Sephadex with CBP, and the use of an eluting buffer containing 2% HS.

Table 3-2. The calculation of the concentration of ^{125}I -11S IgA following purification on a Sepharose 4B column

Purification of 16.87 ug protein from the iodination of 11S IgA
(see Table 2-1).

Total number of fractions collected = 120.

Total counts in all fractions = 53,528 cts/10 secs*

Total counts in peak II fractions pooled for use in RIA
= 13759 cts/10 secs

$$\begin{aligned} \therefore \% \text{ of counts in peak II fractions pooled for use in RIA} \\ &= \frac{13759}{53528} \times 100 \\ &= 25.7\% \end{aligned}$$

volume of peak II fractions pooled = 21 ml.

As the amount of protein used initially was 16.87 ug,

$$\begin{aligned} \text{Concentration of } ^{125}\text{I-11S IgA} &= \frac{16.87 \times 0.257}{21} \\ &= 0.216 \text{ ug/ml} \end{aligned}$$

* The fractions from Sepharose 4B gel filtration were counted with a different geometry from the fractions from Sephadex G50 gel filtration (Table 2-1).

Calculation of the concentration of iodinated immunoglobulin following Sepharose 4B purification

Sepharose 4B was routinely used for the purification of ^{125}I -11S IgA and ^{125}I -7S IgA from Sephadex G50 gel filtration. After the determination of the radioactivity present in each fraction arising from Sepharose 4B gel filtration, the following calculation was made to determine the concentration of iodinated immunoglobulin present in the fractions containing tracer for use in RIA, and is illustrated in Table 2-2.

(a) The total radioactivity was determined in all the Sepharose 4B gel filtration fractions.

(b) The radioactivity present in the seven fractions with the maximal amounts of radioactivity was calculated.

(c) The value for (b) was then divided by (a) to calculate the concentration of iodinated immunoglobulin in the pool of seven fractions collected.

In this calculation, no allowance is made for the quantity of radioactivity left in the column, or in test tubes, but the losses are again minimised by the use of the eluting buffer containing 2% HS.

Properties of Iodinated Immunoglobulins

Gel Filtration: Sephadex G50

An elution profile of the reaction mixture following Chloramine T iodination showed two peaks of radioactivity: a first peak, eluting at the void volume, containing iodinated proteins (Peak I, Fig. 3.1) and a second peak (Peak II) containing free ^{125}I . The data from the Sephadex G50 elution profile was used to calculate the specific activity of iodinated immunoglobulin, and the yield of the iodination reaction, as described above.

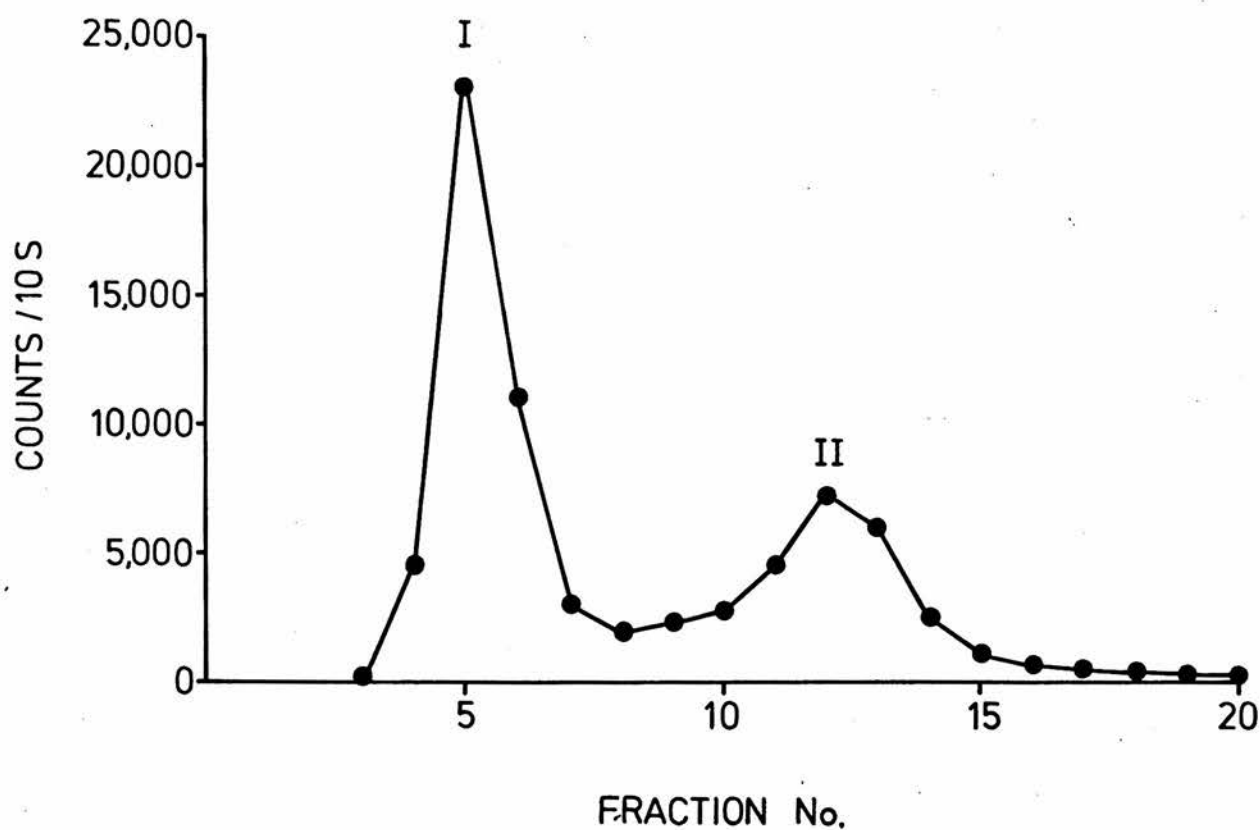


Fig. 3.1:

Elution profile of iodinated protein mixture following reaction of Na^{125}I with 11S IgA, when purified on a Sephadex G50 column. Two peaks of radioactivity were observed: ^{125}I -protein (Peak I) and Na^{125}I (Peak II). Procedural details are described in the text.

Sepharose 4B

The use of Sepharose 4B for gel filtration permits the separation of proteins with molecular weights less than 20×10^6 daltons, and is therefore suitable for studying protein mixtures likely to contain immunoglobulin aggregates or small peptide fragments resulting from the iodination method.

(a) 11S IgA: a typical radioactivity elution profile from Sepharose 4B gel filtration of the fraction from Sephadex G50 purification containing the maximal radioactivity in the iodinated protein peak (e.g. fraction no 5, Table 2-1) is shown in Fig. 3.2. Three peaks were observed: a small peak, eluting at the void volume (Peak I), and presumably consisting of aggregated iodinated immunoglobulin; a large peak (Peak II), eluting at a volume consistent with the molecular weight of unlabelled 11S IgA; and a slightly smaller peak (Peak III), eluting at a volume consistent with a molecular weight of approximately 20,000 daltons (on the basis of marker proteins consisting of thyroglobulin, bovine serum albumin and cytochrome C analysed on the same column). Peak III varied in size from iodination to iodination but never exceeded 20% of the total radioactivity eluting from the column.

(b) 7S IgA: A typical radioactivity elution profile on Sepharose 4B gel filtration of the fraction resulting from Sephadex G50 purification containing maximal radioactivity in the iodinated protein peak is shown in Fig. 3.3. A peak was observed at an elution volume consistent with the molecular weight of unlabelled 7S IgA, and some radioactive protein eluted before the major peak.

(c) IgG: The radioactive elution profile corresponding to that described in (b) above, but for IgG is shown in Fig. 3.3. Only

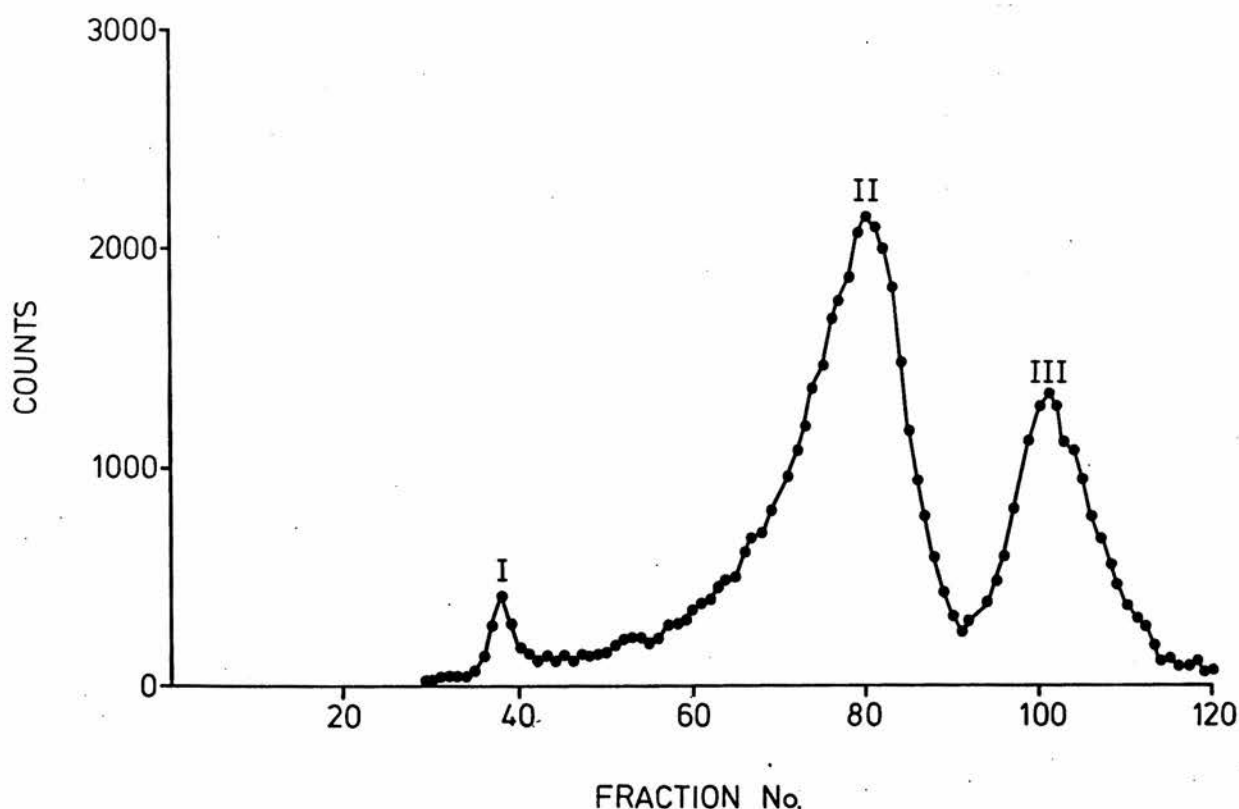


Fig. 3.2:

Elution profile of ^{125}I -protein (from Sephadex G50 purification, see Fig. 3.1) when further purified on a Sepharose 4B column. Three peaks of radioactivity were observed: aggregated ^{125}I -IgA at the void volume (Peak I), ^{125}I -11S IgA (Peak II) and low molecular weight iodinated protein ($\text{MW} \approx 20,000$ daltons, Peak III). Procedural details are described in the text.

one peak was observed at an elution volume consistent with the molecular weight of unlabelled IgG and no other radioactive protein eluted before or after the major peak.

Binding of Iodinated Proteins to Excess Specific Antibody

The appearance of three peaks of radioactivity (Fig. 3.2) in the radioactive elution pattern for 11S IgA indicated further experiments using specific antisera, particularly for Peak III (Fig. 3.2). It was found that Peak I radioactivity was bound poorly by rabbit anti-7S IgA indicating that Peak I material was probably aggregated IgA. With Peak II, radioactivity was bound with anti-7S IgA, anti-light chain antisera (κ and λ) and anti-secretory component, indicating that Peak II contained the complete 11S IgA molecule. However, Peak III radioactivity was not bound by any of the antisera used, indicating that Peak III probably represented some unknown peptide fragment without any antigenic determinant known to be present on the intact 11S IgA or 7S IgA molecule (Table 3-3).

These studies were not carried out with 7S IgA or IgG as only one major peak of radioactivity was observed in both cases.

Direct measurement of labelled and unlabelled immunoglobulins by RIA

^{125}I -11S IgA : Complete superimposability was seen for the respective curves for unlabelled 11S IgA and ^{125}I -11S IgA (Fig. 3.4) indicating that the Peak II material (representing purified ^{125}I -11S IgA) from Sepharose 4B gel filtration was not detectably damaged by the iodination procedure.

^{125}I -7S IgA: When a graph corresponding to Figure 3.4 was plotted for ^{125}I -7S IgA and unlabelled 7S IgA, it was found that the graph for ^{125}I -7S IgA was parallel but slightly to the left of the graph for unlabelled 7S IgA (not shown). This was due to the fact that the

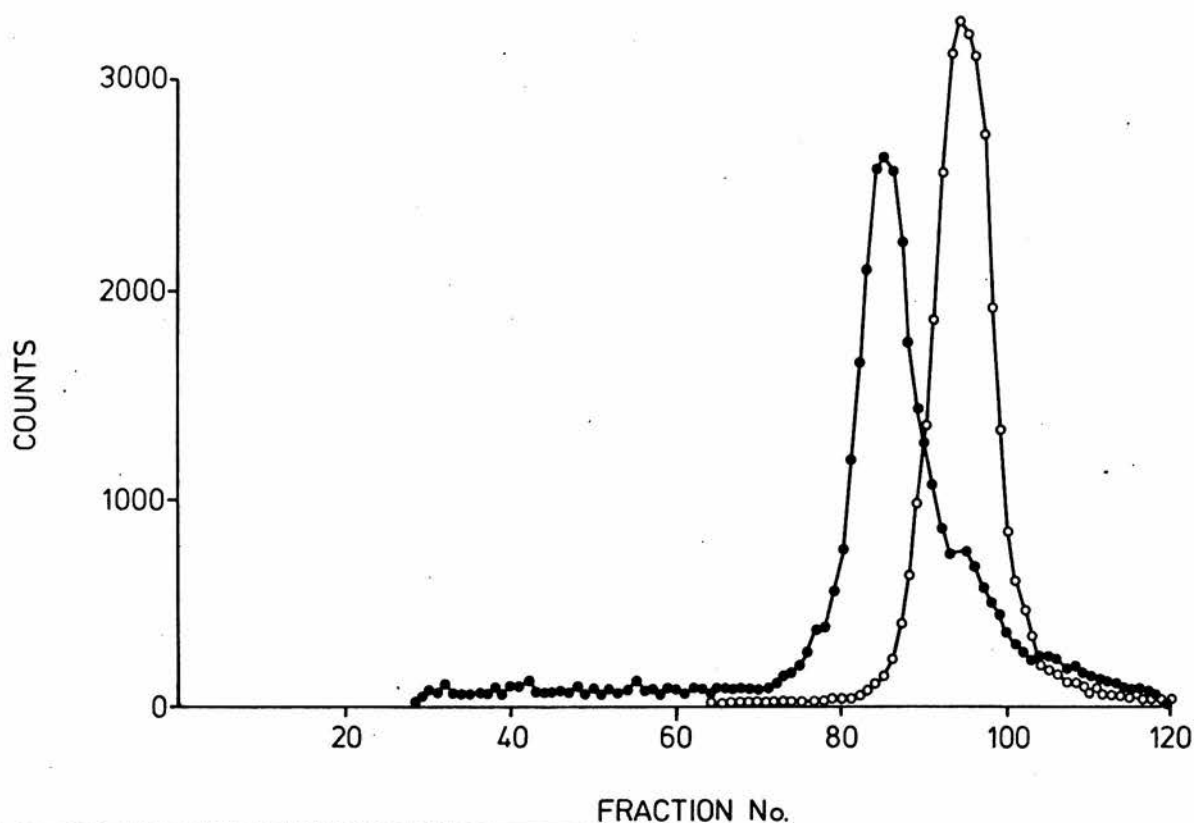


Fig. 3.3:

Elution profile for ^{125}I -7S IgA (●—●) and ^{125}I -IgG (○—○) (from Sephadex G50 purification) when further purified on a Sepharose 4B column. In both cases, only one major peak of radioactivity was observed. Procedural details are given in the text.

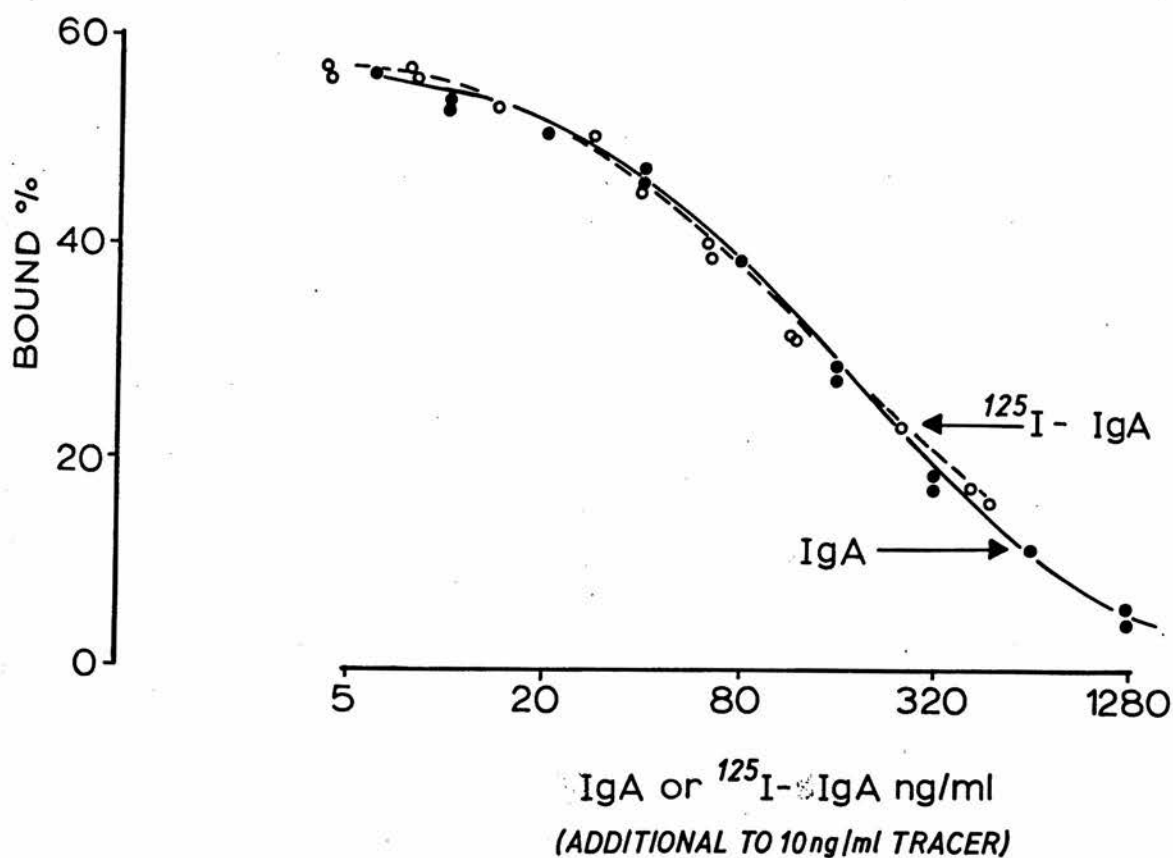


Fig. 3.4:

Assessment of the immunological activity of purified ^{125}I -11S IgA. A standard curve consisting of increasing amounts of unlabelled 11S IgA ($\bullet\text{---}\bullet$) was compared with a curve of increasing calculated amounts of ^{125}I -11S IgA ($\circ\text{---}\circ$). After separation of bound from free protein, the percentage of original added counts bound to antibody was calculated, and the two curves tested for superimposability.

protein concentration value for the labelled 7S IgA was obtained by direct protein measurement (Schuurman, 1977) whereas the concentration value for the unlabelled 7S IgA was based upon the W.H.O. International Standard (see Chapter 4.). It was therefore considered unlikely that damage had occurred during iodination of 7S IgA.

^{125}I -IgG: When a graph corresponding to Figure 3.4 was plotted for 7S IgG and ^{125}I -7S IgG, complete superimposability was found (not shown) indicating that no immunologically detectable damage had occurred to 7S IgG during the iodination procedure.

3.4 DISCUSSION

The purpose of the experiments described in this chapter was to assess the use of the Chloramine T method for producing iodinated immunoglobulins for RIA. The quality of iodinated immunoglobulins can be affected in three different ways: firstly, the insertion of a ^{125}I iodine atom into the immunoglobulin molecule, or the chemical reagents used in iodination can damage the immunoglobulin molecule by altering the protein structure. Primary radiation damage can also occur from γ -emission by the ^{125}I incorporated into the immunoglobulin molecule. Secondly, variations in the stability of the protein to be iodinated can affect the quality of the ^{125}I -labelled product. Thirdly, the method of purifying ^{125}I -labelled protein can affect the quality of the tracer.

The Chloramine T method was found to be convenient and satisfactory; once the optimum quantities of radioiodine to be used had been ascertained, high yields (65-85%) of iodinated immunoglobulins were produced with specific activities representing the incorporation of about one atom of ^{125}I per molecule of immunoglobulin. However, of

Table 3-3 Analysis of Peaks I, II and III from Sepharose 4B
gel filtration of iodinated protein from 11S IgA
iodination

<u>Antiserum</u>	<u>Binding %</u>		
	<u>Peak I</u>	<u>Peak II</u>	<u>Peak III</u>
Anti-7S IgA	19	97	
Anti-secretory component	-	89	
Anti- κ light chain	-	56	
Anti- λ light chain	-	40	less than
Anti-lactoferrin	-	-	2%*
Anti-whole colostrum	-	-	
Anti-whole serum	-	-	

All antisera were obtained from Dakopatts except for anti-7S IgA, anti-lactoferrin and anti-whole serum, which were obtained from T.A.E. Platts-Mills, R.R. Samson and the Dutch Red Cross Blood Transfusion Service respectively.

* Non-specific binding averaged 2% in this experiment.

the three immunoglobulins that were iodinated, problems were encountered with 11S IgA, which required further purification by gel filtration using Sepharose 4B. As can be seen in the elution profile of radioactivity from Sepharose 4B gel filtration of iodinated 11S IgA (Fig. 3.2); three types of iodinated proteins were produced, only one of which was bound completely by specific antisera directed against antigenic determinant present in the complete unlabelled 11S IgA molecule. This material (Peak II, Fig. 3.2) was used as tracer for 11S IgA RIA. The iodinated protein eluting at the void volume probably represented aggregated 11S IgA, as iodination of prolactin also results in the formation of high molecular weight aggregates (Sharpe, 1979). It was not possible to identify the low molecular weight material (Peak III, Fig. 3.2) and it is probable that this peak consists of a portion of the 11S IgA molecule cleaved during iodination, losing all antigenic determinants in the process. It was therefore decided that for 11S IgA RIA, purification of the iodinated protein material required both Sephadex G50 and Sepharose 4B gel filtration. The quality of the tracer purified by this method is shown by the complete superimposability of the standard curve for labelled and unlabelled 11S IgA (Fig. 3.4).

A similar purification using Sepharose 4B for the labelled protein resulting from iodination of 7S IgA was undertaken as some slight impurities were present (Fig. 3.3). By contrast, as the labelled protein resulting from iodination of IgG was considered to be pure (Fig. 3.3) and immunologically undamaged, purification with Sepharose 4B was not undertaken.

CHAPTER 4

RADIOIMMUNOASSAY AND STATISTICAL
METHODS

4.1 INTRODUCTION

In Radioimmunoassay (RIA), a fixed concentration of labelled antigen (tracer) is incubated with a constant dilution of specific antiserum such that the concentration of antigen binding sites on the antibody is limiting. If unlabelled antigen is added, competition occurs between tracer and unlabelled antigen for the limited and constant number of antibody binding sites, and the amount of tracer bound to antibody will decrease as the concentration of unlabelled antigen increases. This can be measured by the appropriate separation procedure (e.g. the double antibody method used in this thesis). A standard curve is therefore set up with increasing concentrations of standard unlabelled antigen, and from this curve the amount of antigen in unknown samples can be calculated. In a number of studies described in this thesis, concentrations of 11S IgA, 7S IgA and IgG (acting as antigen not antibody) have been determined using specific RIA. The following is an account of the reagents and methodology used.

4.2 MATERIALS AND METHODS

4.2.1 Materials

All antisera, standards, second antibody precipitating sera, quality control samples and non-immune rabbit carrier sera were stored in separate aliquots in stoppered 63 x 11 mm polystyrene tubes at -20°C till use, to avoid freezing and thawing each solution more than once.

(a) Standards

All standards were kept at three concentrations varying eight-fold in concentration.

11S IgA: As no International Reference Preparation for this protein exists, the purified 11S IgA preparation described previously (Chapter 3) was used as a standard. The protein value for this standard was obtained by a determination of dry weight by Mr. R.R. Samson.

7S IgA and IgG A commercial standard (Hyland) that had previously been standardised against the W.H.O. Standard (Rowe, Anderson and Grab, 1970) was used.

(b) Antisera

Anti-human 7S IgA and anti-human IgG specific for heavy chain antigenic determinants (Platts-Mills and Ishizaka, 1975) were used for 11S IgA, 7S IgA and IgG RIA. These antisera were kindly given by Dr. T.A.E. Platts-Mills.

An antiserum specific for the conformational determinant of 11S IgA ('C', shown diagrammatically in Fig 4.10) and for the accessible determinant present on free secretory component ('A') was prepared by two further adsorptions of rabbit anti-11S IgA/FSC (IOMSP, Dakopatts) with 7S IgA coupled to Sepharose 4B.

(c) Sera for precipitation of first antibody

Donkey anti-rabbit serum (Wellcome; Scottish Antibody Production Unit) and non-immune rabbit serum (Wellcome) acting as carrier, were used for precipitation in all RIA. For the IgG RIA only, donkey anti-rabbit serum that had previously been adsorbed with human IgG coupled to Sepharose was used. The adsorption procedure was used to remove all donkey antibodies that reacted with antigenic determinants that were common to human IgG and rabbit IgG, thus allowing separation of

human IgG (antigen) bound to rabbit IgG (antibody), from free human IgG.

Each batch of donkey anti-rabbit serum was optimised prior to use with normal rabbit carrier serum, to ensure maximal precipitation of (rabbit) antibody bound immunoglobulin as well as economical use of precipitating sera.

(d) Assay buffer

The assay buffer used in all instances was 0.05 M Na_2PO_4 buffer at pH 7.5, containing 2% horse serum. In assays with longer incubation periods, or where sucrose was present (from ultracentrifugation studies), 0.01% (w/v) NaN_3 was included in the buffer.

4.2.2. Methods

(a) Preparation of immunoadsorbents

Sepharose 4B (37.5 gm; Pharmacia), washed thoroughly with distilled water, was activated using the method of Axen, Porath and Ernbeck (1967) by addition of cyanogen bromide (4g; CNBr; Aldridge) at pH 10.5 in a well ventilated fume cupboard. The activated CNBr was then washed with 0.1 M NaHCO_3 (2 l) and 0.1 M sodium citrate solution pH 6.0 (2 l), then mixed with human IgG (750 mg; Protein Fractionation Centre, Edinburgh), or undiluted serum (2 ml, from three patients with IgA myelomas, courtesy of Dr. D.B.L. McClelland), and the mixture diluted in citrate buffer to 50 ml total volume. Coupling of protein to activated CNBr occurred over 18 h at 4°C. The coupled protein was then washed thoroughly (2 l each of 0.1 M NaHCO_3 , 0.05 M Na_2PO_4 pH 7.5, and 0.05 M Na_2PO_4 pH 7.5 containing 2% horse serum), prior to use as an immunoadsorbent. The Sepharose-IgG preparation was sufficient to adsorb 80-100 ml DARS; the Sepharose-serum IgA

preparation was sufficient to adsorb 2 ml of rabbit anti-11S IgA/FSC.

(b) RIA procedure

The standard double antibody technique was used in all RIA. Known volumes of mammary secretions, sera, culture fluids or fractions from gel filtration and ultracentrifugation, or the appropriate immunoglobulin standard (using a X2 concentration interval) were diluted to a total volume of 0.2 ml in assay buffer. The specific antiserum (first antibody) was added in a volume of 0.1 ml, and the mixture incubated for three hours in 63 x 11 mm polystyrene tubes at room temperature. Labelled immunoglobulin was then added in a volume of 0.1 ml, and incubation allowed to proceed for a further three hours, following which donkey anti-rabbit serum (0.05 ml) and normal rabbit serum (0.05 ml) were added at the appropriate dilutions. Precipitation of (rabbit) antibody bound immunoglobulin occurred over 12 - 16 h at 4°C, following which bound tracer was separated from free tracer by centrifugation (1,500 x g for 30 min., 4°C) after addition of 1.5 ml assay buffer to all assay tubes (except total count tubes). The radioactivity in the precipitate was then counted in a γ - spectrometer (LKB; Nuclear Enterprises).

In the IgG RIA only, antiserum and ^{125}I -IgG tracer were added in a volume of 0.05 ml instead of 0.1 ml to reduce the amount of antiserum used. Assays which required a higher degree of sensitivity than could be attained by the usual procedure were performed using a 16 and 8 hour incubation period before and after addition of tracer respectively instead of two incubation periods of 3 hours duration.

Incorporated into the above procedure were tubes which contained either:

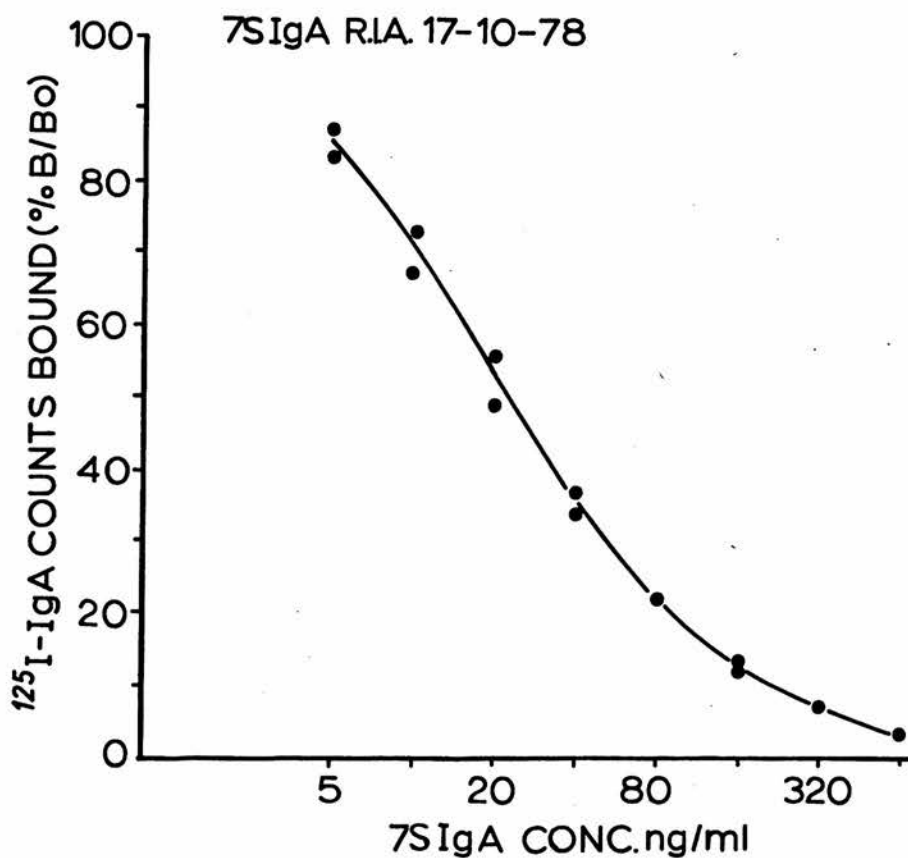
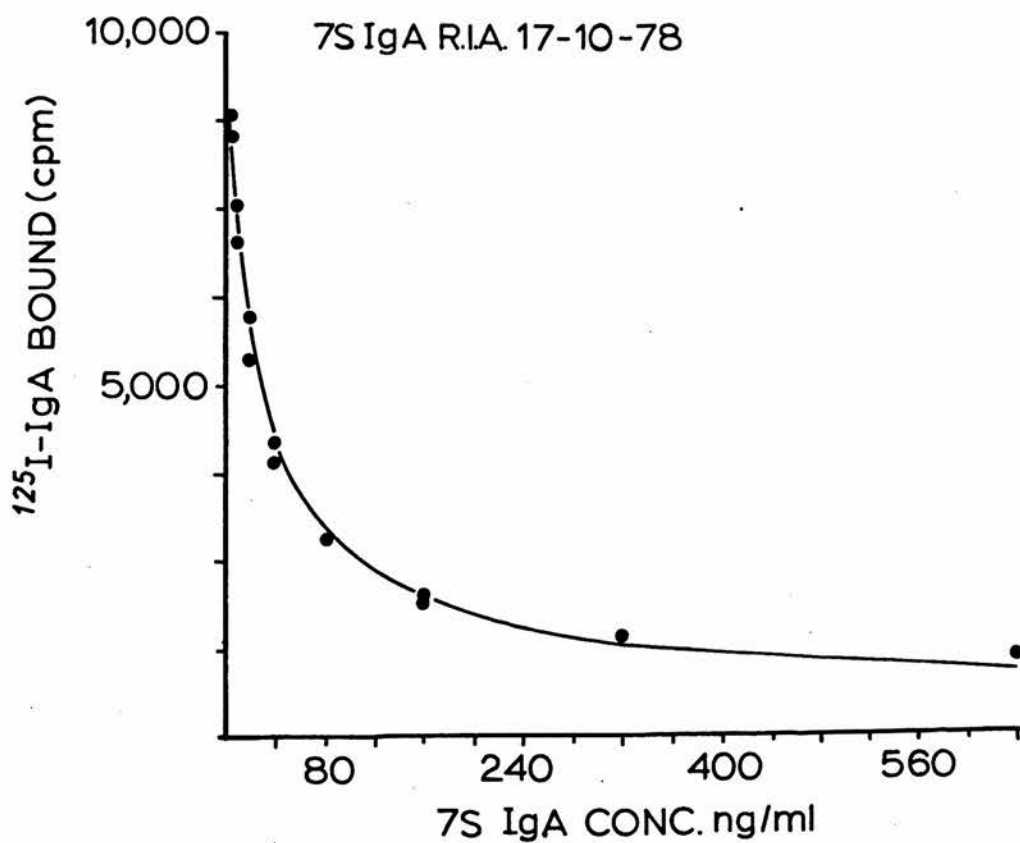


Fig. 4.1:
 Analysis of RIA Standard Curve Data.
 Above: Raw counts vs. IgA concentration
 Below: % B/Bo vs log IgA concentration.

- (a) assay buffer + ^{125}I -labelled immunoglobulin
- (b) assay buffer + ^{125}I -labelled immunoglobulin + first antibody (specific antiserum)
- (c) ^{125}I -labelled immunoglobulin only.

These tubes were used to assess

(a) Non-specific binding (NSB) i.e. the binding of ^{125}I -labelled immunoglobulin in the absence of first antibody (specific antiserum).

(b) the zero binding or B_0 i.e. the maximal binding of ^{125}I -labelled immunoglobulin to the particular concentration of first antibody used in the assay

(c) the amount of radioactivity (i.e. labelled immunoglobulin) added per tube = total counts.

All experimental samples, standards and tubes for the assessment of total counts, NSB and B_0 were run in duplicate.

An automatic diluting and dispensing system (Compupet, General Diagnostics) or precision dispensing pipettes (Eppendorf; Finnpiquette) were used for the addition of test fluids, standards and diluent buffers. Additions of first antibody, labelled immunoglobulin, second antibody and rabbit carrier serum were made with the Compupet, or a 2.5 or 5 ml graduated glass syringe with an attached repeating dispenser (PB 600-1, Hamilton). The 1.5 ml volume assay buffer used to dilute incubates prior to centrifugation was added with an adjustable dispenser (Lumomix).

(c) Assay Analysis

The analysis of RIA data was performed automatically using a programme written by Dr. R.M. Sharpe for a Hewlett-Packard desk-top

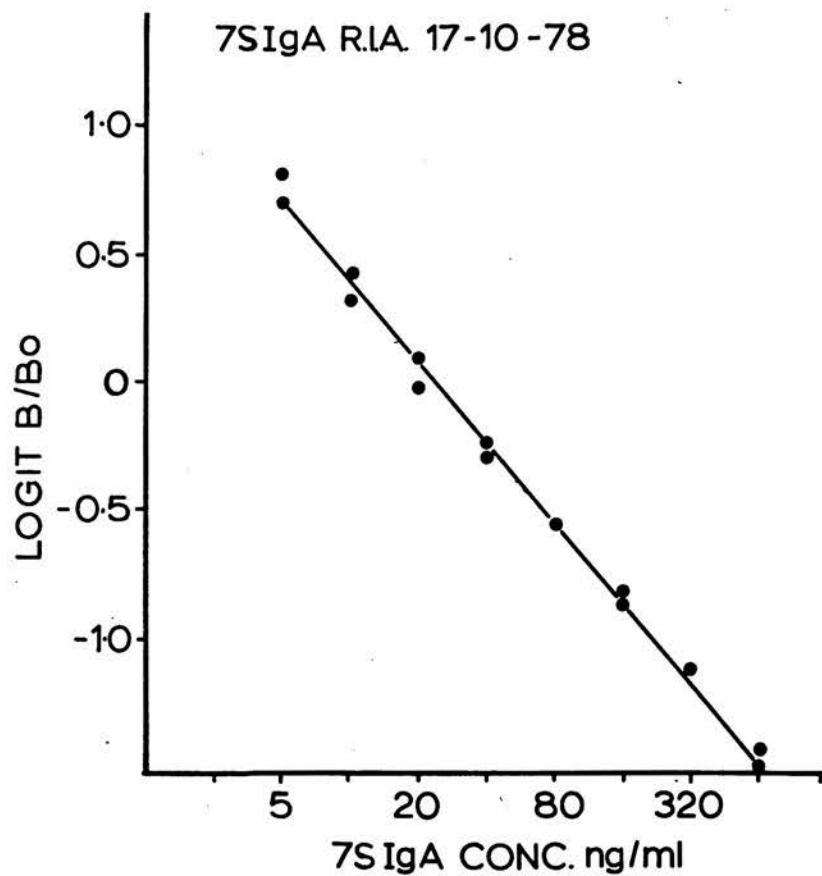


Fig. 4.1 (cont.)

Analysis of RIA Standard Curve Data
Logit B/Bo vs log IgA concentration.

calculator (model 9821A). This programme subtracted the mean cpm radioactivity in the NSB tubes from the cpm in all other tubes (excluding total counts) and the remaining radioactivity was considered as specifically bound to the first antibody. For each point on the standard curve, the cpm bound in duplicate tubes was expressed as a % of that bound in the B_0 tubes (i.e. B/B_0), and a standard curve constructed (e.g. Fig. 4.1). In order to allow a straight line equation to be fitted to the results, the percentages were transformed to logits. Using the method of least squares, the straight (regression) line of best fit was then calculated using data for standards that were within the range 10-90% B/B_0 ; this restriction was imposed due to the variability of the results approaching the asymptotes.

The programme then ascertained that the straight line fitted to the data was in fact the 'best' line: the mean cpm for each point on the standard curve was re-entered, and, using the 'best-fit' lines the amount of immunoglobulin present calculated. This value was then compared with the amount of immunoglobulin added. If the known and calculated amount of immunoglobulin differed by 30% or more, then that particular point on the standard curve was omitted, the 'best-fit' straight line recalculated, and the above procedure repeated. Subsequently, the same procedure was used to locate (and discard) points with errors of 20%, then of 10%. Once the final 'best fit' line was achieved, the transformed data for all samples was substituted into the equation for this line, and the immunoglobulin present in the sample calculated and printed out. Values for samples outwith the 10-90% B/B_0 range were not calculated and the samples were subsequently re-assayed at a different dilution. Visual confirmation that the above

procedures were accurate was provided in every instance by a simultaneous plot of the standard curve data by an automatic X-Y graph plotter (Hewlett-Packard) attached to the calculator.

(d) Validation of RIA

Validation of the specificity of each RIA used was required because of the common antigenic determinants that appear in the immunoglobulins measured e.g. 11S IgA, 7S IgA and IgG share common light chain antigens; 11S IgA and 7S IgA share common heavy α -chain antigens.

Validation was performed in the following manner:

- (i) The possibility of IgG interfering in IgA RIA, and IgA interfering in IgG RIA was determined.
- (ii) The use of ^{125}I -11S IgA instead of ^{125}I -7S IgA tracer in measuring IgA in serum was examined. IgA concentrations in the sera of each of 55 normal subjects were determined by RIA using either ^{125}I -11S IgA or ^{125}I -7S IgA as tracer, with the same 7S IgA standard and anti-7S IgA antiserum.
- (iii) RIA was compared with two widely used methods for the measurement of immunoglobulins: single radial immunodiffusion (SRID; Fahey and McKelvey, 1965) and laser nephelometry. Using the same 7S IgA and IgG standards, serum IgA and IgG concentrations were determined in 53 and 60 normal sera respectively by either RIA, commercial immunodiffusion plates (Hyland, or Behring LC-Partigen) or a Hyland laser nephelometer with sheep anti-7S IgA or sheep anti-IgG (Seward) as specific antiserum.

Using the same 11S IgA standard, 11S IgA concentrations were determined in 48 colostrum and milk samples by either RIA or single radial immunodiffusion plates prepared in the Department of Therapeutics

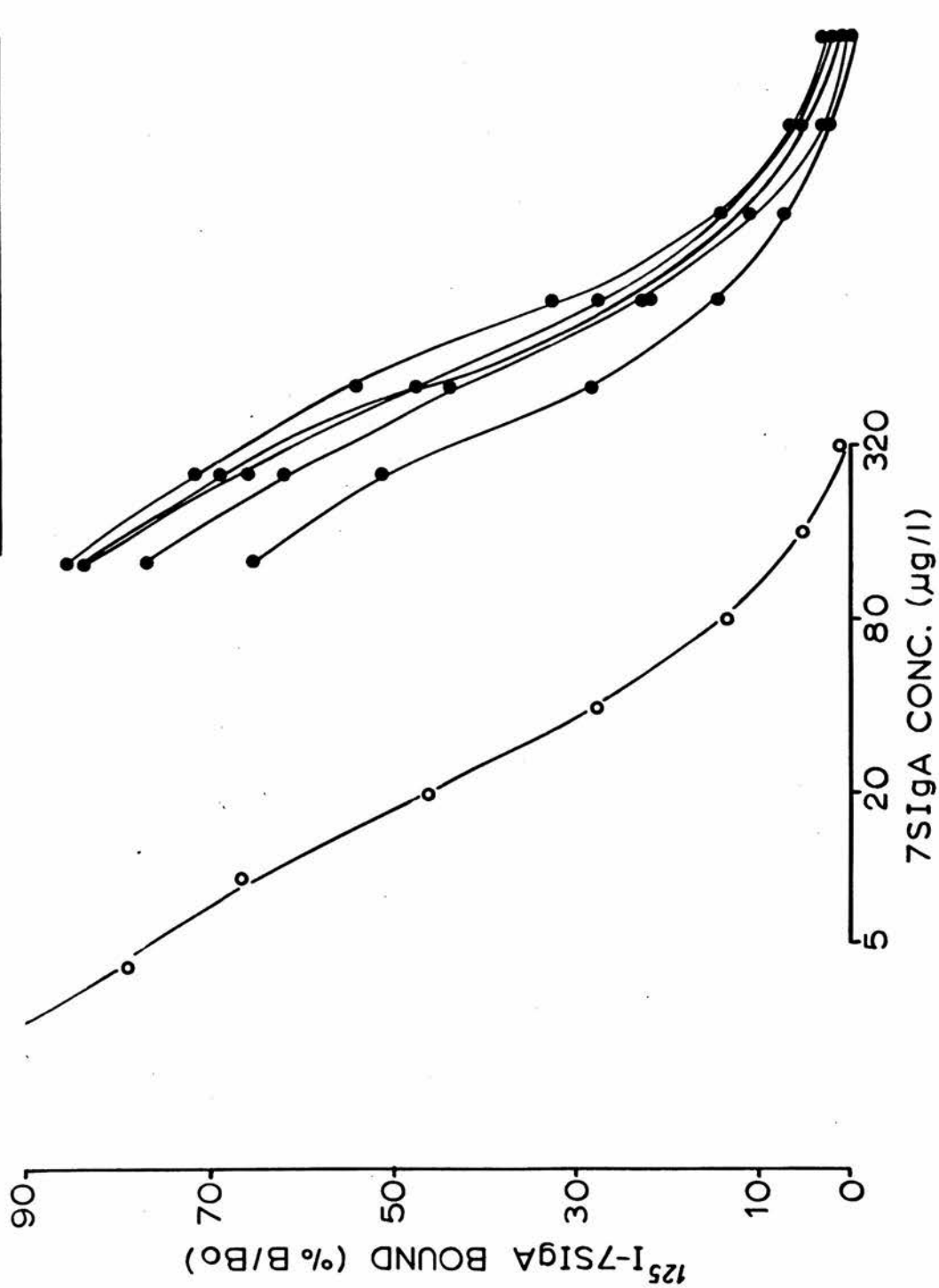


Fig. 4.2: Inhibition curves for 7S IgA standard (o---o) and five normal adult sera (•—•). In this assay, ^{125}I -7S IgA was used as tracer, and rabbit anti-human 7S IgA as antiserum.

using goat anti-7S IgA (Hyland). The correlation coefficient was determined for a comparison of values obtained by RIA with those obtained by either SRID or nephelometry. A paired Student's t-test was used to determine if the value for each serum was significantly different from the value for the same serum obtained by SRID and nephelometry.

(iv) Gel filtration using a Sepharose 4B column (900 x 22 mm) was carried out on samples of colostrum, milk and normal adult serum; 120 fractions of 3 ml volume were collected using downward flow of eluate (RIA buffer) and stored at -20°C till immunoassay for 11S IgA, 7S IgA or IgG without concentration.

(v) The precision and reproducibility (as defined by Whitehead, 1977) of the RIA was determined in the following manner: for the determination of intra-assay CV, the same sample was analysed at different concentrations 20 times in RIA; for the determination of inter-assay CV, the sample was assayed at different concentrations in 10 assays in duplicate (7 assays for IgG RIA and 7S IgA RIA using ^{125}I -7S IgA), by placing the sample tubes at the 'end' of the assay.

In both cases, the sample used was derived from aliquots of the appropriate standard (7S IgA, 11S IgA or IgG) and stored at -20°C till assay.

4.3 RESULTS

In assessing the use of RIA for the measurement of immunoglobulins, the parallelism of inhibition of binding by the immunoglobulin standard, and various samples was first determined. It was found that the binding of ^{125}I -11S IgA to rabbit anti-human 7S IgA was displaced in a parallel manner by the 11S IgA standard, and by various samples of

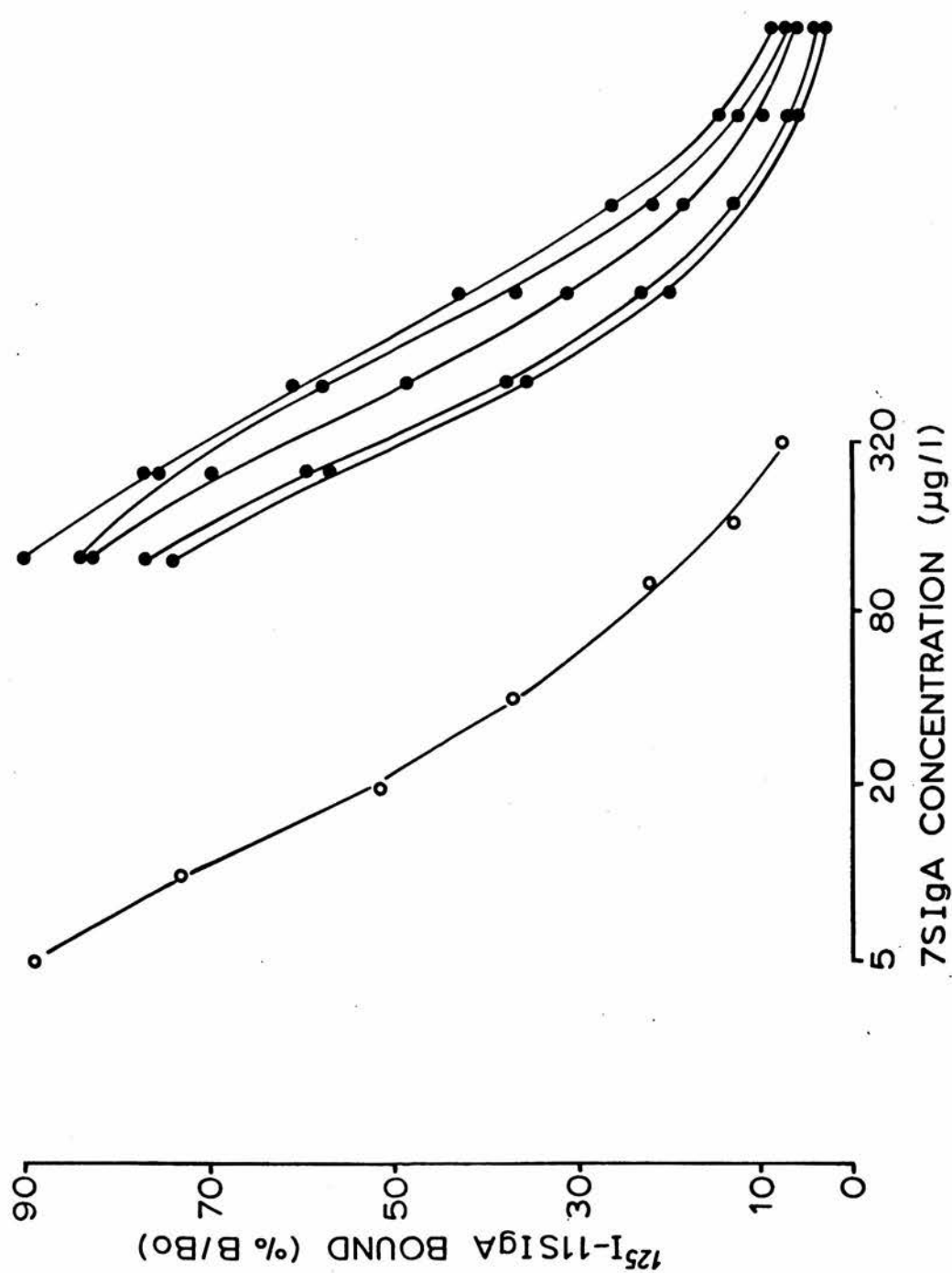


Fig. 4.3:
Inhibition curves for 7S IgA standard (o—o) and five normal adult sera (•—•).
In this assay, ^{125}I -11S IgA was used as tracer, and rabbit anti-human 7S IgA as antiserum.

colostrum or milk, in which 11S IgA is the predominant form of IgA present (not shown). Similarly, parallel inhibition curves were observed when the 7S IgA standard was compared with various sera (where 7S IgA is the predominant form of IgA present) in an RIA using ^{125}I -7S IgA tracer and rabbit anti-human 7S IgA (Fig. 4.2). Parallel inhibition curves were also observed when the binding of ^{125}I -7S IgG to rabbit anti-human IgG, in the presence of the 7S IgG standard or of various serum samples, was studied (not shown).

Interference in RIA by other immunoglobulins

(i) In an 11S IgA RIA, 50,000 ng of purified IgG produced the same inhibition of binding of ^{125}I -11S IgA as 32.9 ng of purified 11S IgA. Similarly, in an IgG RIA, 2560 ng of purified 11S IgA produced the same inhibition of binding of ^{125}I -IgG as 14 ng of purified IgG.

(ii) Comparison of ^{125}I -11S IgA and ^{125}I -7S IgA tracer for measuring 7S IgA

A validation of the use of ^{125}I -11S IgA tracer for measuring 7S IgA, in combination with a 7S IgA standard and an anti-7S IgA antiserum, was undertaken as it was considered that it would be more convenient if ^{125}I -11S IgA could be used as tracer for both 7S IgA and 11S IgA RIA. As a first step in the validation process, it was confirmed that the binding of ^{125}I -11S IgA by rabbit anti-7S IgA was inhibited in a parallel manner by the 7S IgA standard and five normal sera (Fig. 4.3).

The 7S IgA concentration in 55 normal sera was then determined by RIA using either a ^{125}I -7SIgA tracer or a ^{125}I -11S IgA tracer with a 7S IgA standard and rabbit anti-7S IgA. A highly significant correlation of serum 7S IgA concentrations determined using the two

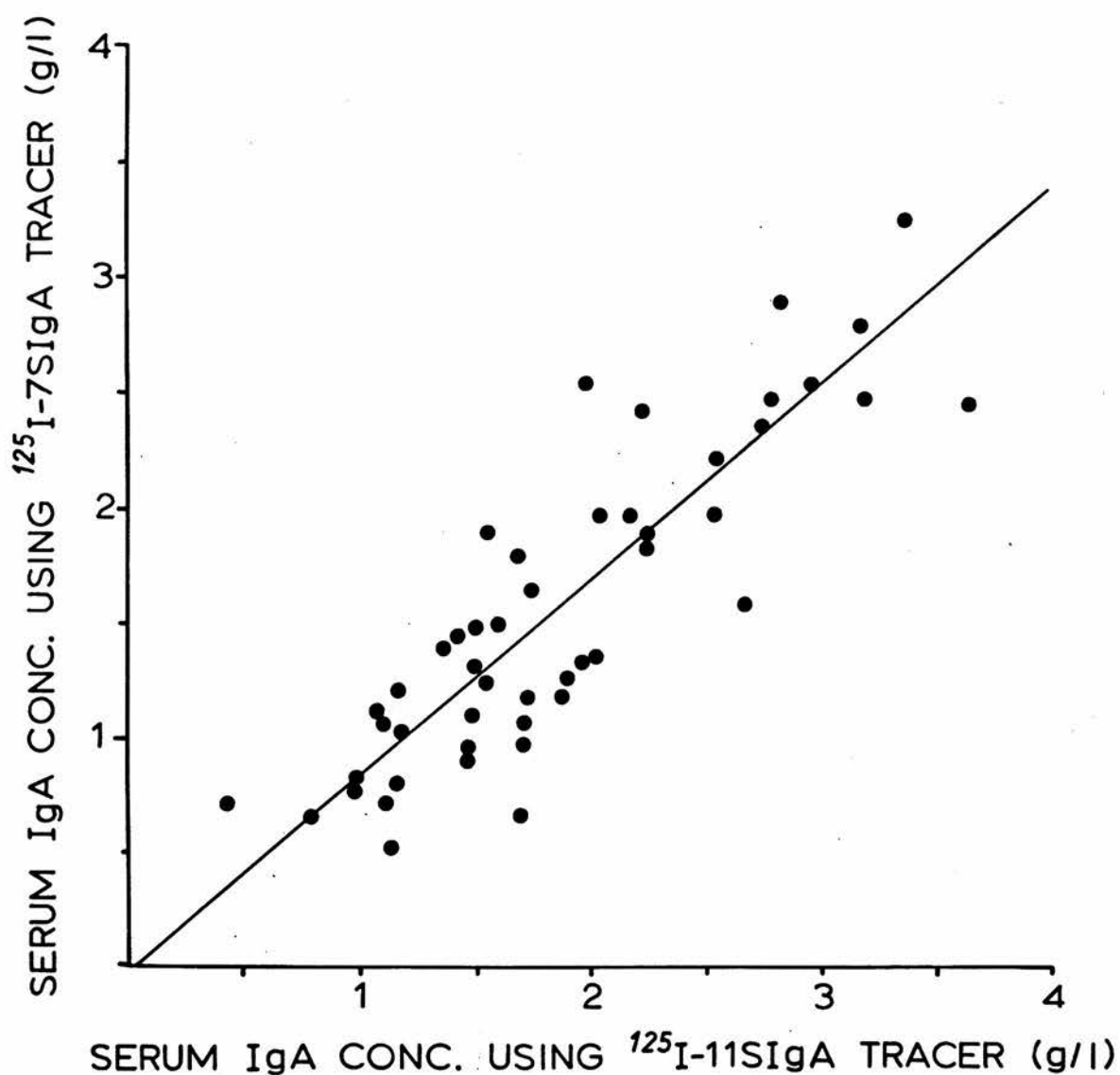


Fig. 4.4:

A comparison of the use of ^{125}I -11S IgA with ^{125}I -7S IgA as tracer in the measurement of serum IgA by RIA (n=55).

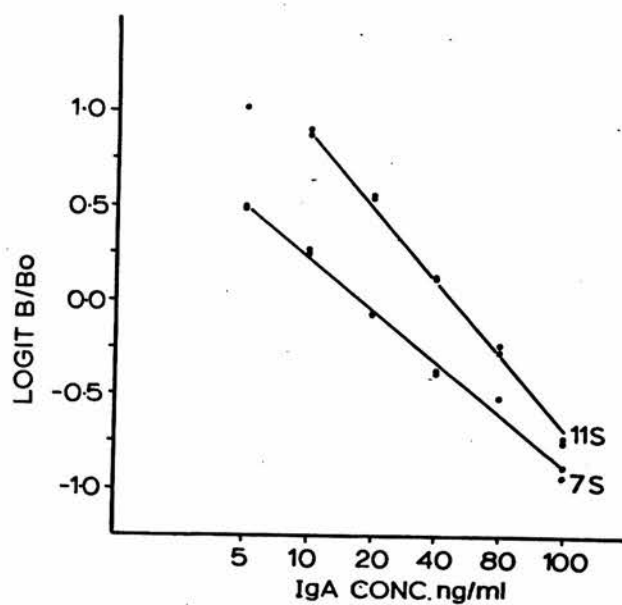
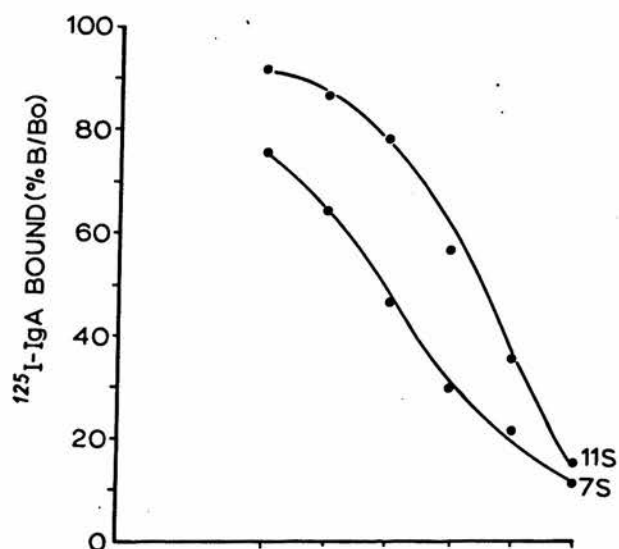


Fig. 4.5:

Non-parallelism in the inhibition of ^{125}I -11S IgA binding between the 11S IgA standard and the 7S IgA standard.

Above: B/Bo versus log IgA concentration.

Below: Logit transformation of same data.

different IgA tracers was found ($r = 0.876$, $p < 0.001$; Fig. 4.4), suggesting that the use of ^{125}I -11S IgA tracer might be valid. However, it was found that the mean 7S IgA value for the 55 sera determined using ^{125}I -11S IgA tracer was 18.5% higher than the mean value found using ^{125}I -7S IgA tracer, a difference that was statistically significant (paired t-test: $t = 6.04$, $p < 0.001$).

It should be noted that the 11S IgA standard and the 7S IgA standard are not interchangeable as they do not inhibit the binding of ^{125}I -11S IgA in a parallel manner. This non-parallelism is more clearly seen when the B/B₀ values undergo a logit transformation (Fig. 4.5).

(iii) Comparison of RIA, SRID and nephelometry for the measurement of immunoglobulins

(a) 11S IgA 11S IgA RIA (using ^{125}I -11S IgA tracer and rabbit anti-7S IgA) was compared with single radial immunodiffusion for measuring 11S IgA in 48 samples of human milk. The same 11S IgA standard was used for both methods and a highly significant correlation was found between the two methods (Table 4-1). The 11S IgA value obtained for each sample by RIA was not significantly different from the 11S IgA value obtained for the same sample by SRID (paired t-test, $t = 1.95$, $p < 0.05$). A correlation of nephelometry with RIA was not performed for the measurement of 11S IgA.

(b) 7S IgA

Initial experiments using ^{125}I -11S IgA tracer together with a 7S IgA standard indicated that it might be possible to measure 7S IgA in serum with this 'heterologous' tracer. Sera from each of 53 normal subjects was therefore measured by either RIA (using ^{125}I -11S IgA tracer and rabbit anti-7S IgA), SRID or laser nephelometry. The same

Table 4-1: A Comparison of RIA, SRID and nephelometry for the measurement of

11S IgA, 7S IgA and IgG

Immunoglobulin	Tracer	Standard	Sample	no.	RIA vs SRID		RIA vs nephelometry	
					r	p	r	p
11S IgA	¹²⁵ I-11S IgA	11S IgA	colostrum milk	48	0.74	<0.001		not done
7S IgA	¹²⁵ I-7S IgA	7S IgA	normal sera	53	0.89	<0.001	0.84	<0.001
IgG	¹²⁵ I-IgG	IgG	normal sera	60	0.80	<0.001	0.85	<0.001

r = correlation coefficient

7S IgA standard was used in all three methods and a highly significant correlation was found when the RIA was compared with nephelometry or SRID (Fig. 4.6, Fig. 4.7, Table 4-1). No significant difference (paired t-test) was found when values for each individual serum measured by the three different methods was compared.

(c) IgG

A comparison similar to that described above for 7S IgA was carried out, and again a highly significant correlation was found between all three methods (Table 4-1). However, the values obtained by RIA for each individual serum sample were significantly higher than the respective values obtained by SRID or nephelometry (Paired t-test; for RIA vs SRID, $t = 5.36$, $p < 0.001$; for RIA vs nephelometry, $t = 6.17$, $p < 0.001$).

(iv) Gel filtration studies

In the gel filtration studies described in this section, the characteristics of the particular Sepharose 4B column used were defined by the use of three marker proteins of known molecular weight: thyroglobulin, bovine serum albumin and cytochrome C. In all cases, the elution volume (V_e) and void volume (V_o) were determined (using a Blue Dextran marker) and a linear plot made of V_e/V_o against log molecular weight. This calibration graph was used to determine the approximate molecular weight of the peaks of immunoreactivity described below.

(a) Colostrum and milk

When fractions from Sepharose 4B gel filtration of colostrum and milk were subjected to 11S IgA RIA, only single peaks of immunoreactivity were seen. The two peaks overlapped, and the elution volume was consistent with a protein of molecular weight 400,000, thus confirming that 11S IgA was being measured by RIA (Fig. 4.8).

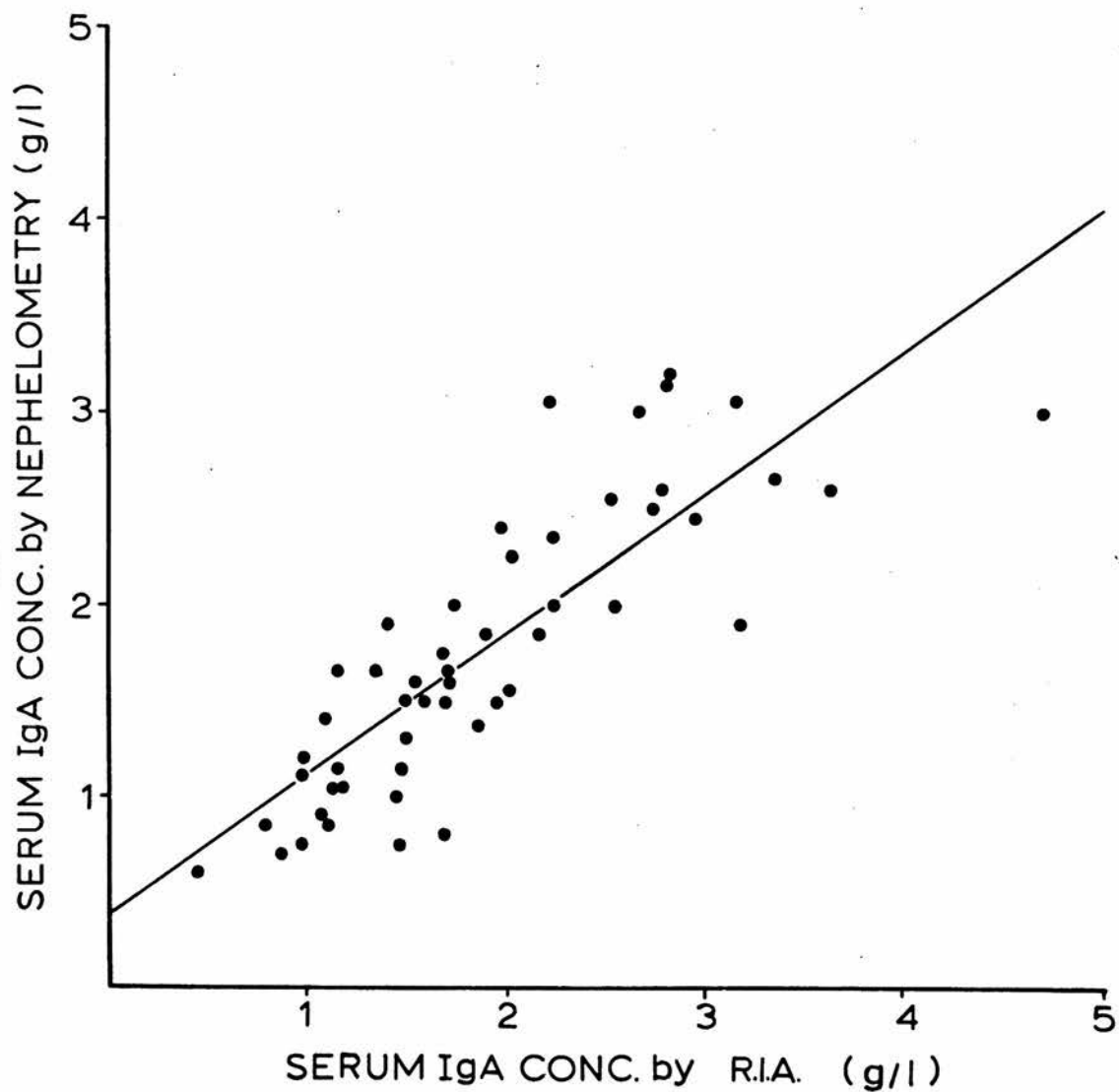


Fig. 4.6:

A comparison of RIA (using ^{125}I -11S IgA tracer) with nephelometry for the measurement of serum IgA (n=53).

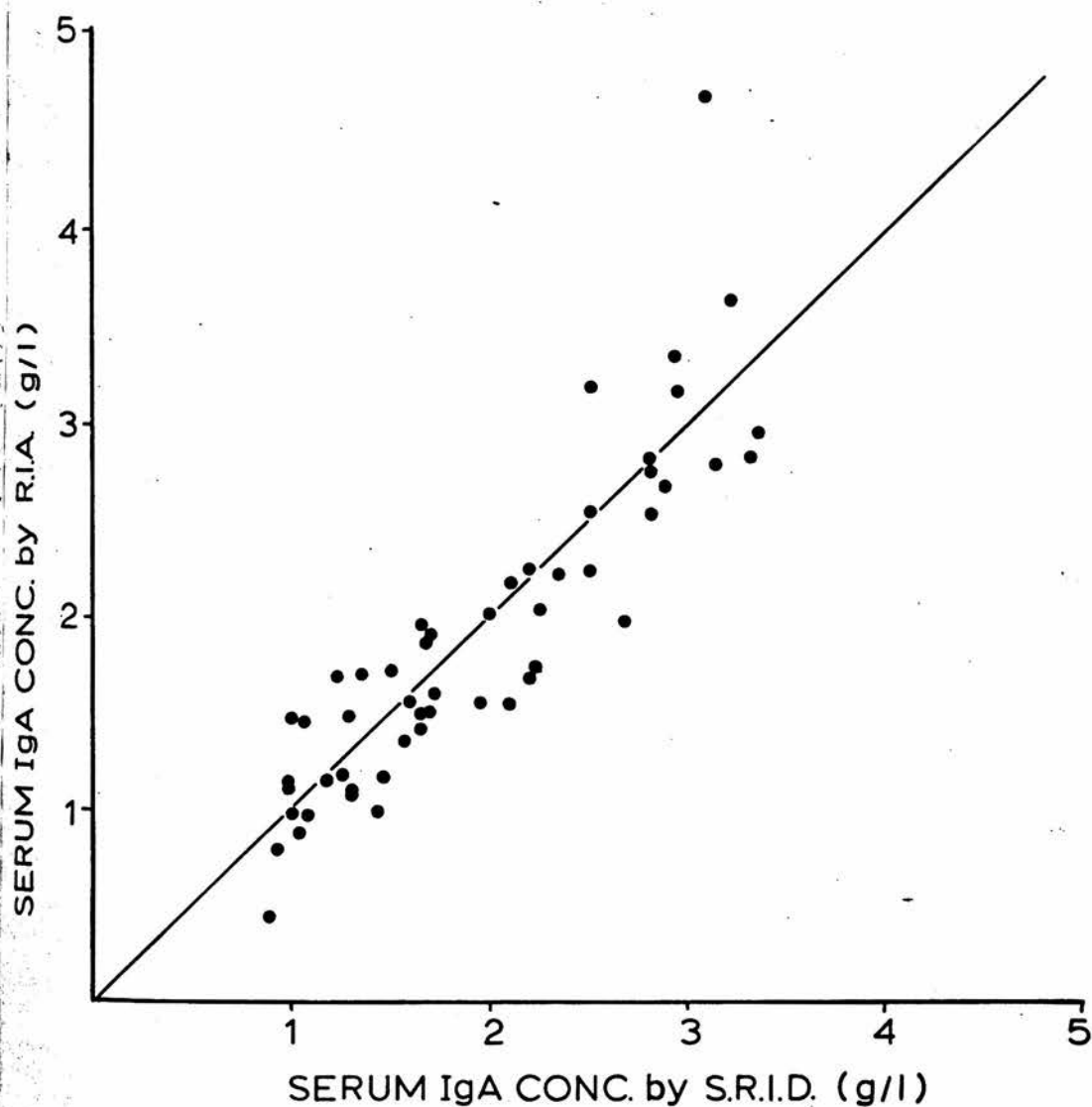


Fig. 4.7:

A comparison of RIA (using ^{125}I -11S IgA tracer) with single radial immunodiffusion (S.R.I.D.) for the measurement of serum IgA (n=53).

(b) Normal serum

When fractions from Sepharose 4B gel filtration of normal adult serum were subjected to 7S IgA RIA (using ^{125}I -11S IgA tracer, 7S IgA standard and rabbit anti-7S IgA), again only one peak of immunoreactivity was seen. However, the elution volume of this peak was consistent with a protein of molecular weight 150,000, confirming that 7S IgA was being measured by the RIA (Fig. 4.9).

In contrast, when the same fractions were assayed by IgG RIA, another peak of immunoreactivity was seen eluting slightly after the 7S IgA peak described above (not shown). The elution volume of this peak is consistent with the finding that the molecular weight of IgG is slightly less than that of 7S IgA and therefore validating the IgG RIA.

Finally, when the same fractions from Sepharose 4B gel filtration of adult serum were immunoassayed using rabbit anti-11S IgA/FSC (following extensive adsorption with serum IgA) in combination with ^{125}I -11S IgA tracer and an 11S IgA standard, no peaks of immunoreactivity were observed (not shown for Sepharose 4B but see Fig.

6.4), particularly in the 7S IgA position, indicating that the antiserum no longer possessed any antibody activity against 7S IgA. Traces of immunoreactive material were seen before the elution volume of 11S IgA, possibly indicating the presence of high molecular weight material with 11S IgA antigenic determinants.

(v) Precision and Reproducibility of RIA

(a) For the determination of 11S IgA, the intra-assay CV for samples with mean 11S IgA concentrations of 18.6, 36.1, 77.8 and 171.8 ng/ml was 15.6, 6.2, 6.9 and 5.8% respectively. The inter-assay CV for samples with mean 11S IgA concentrations of 12.5, 21.4, 42.3, 83.3 and 175.2 ng/ml was 13.4, 7.6, 9.7, 6.9 and 5.3% respectively.

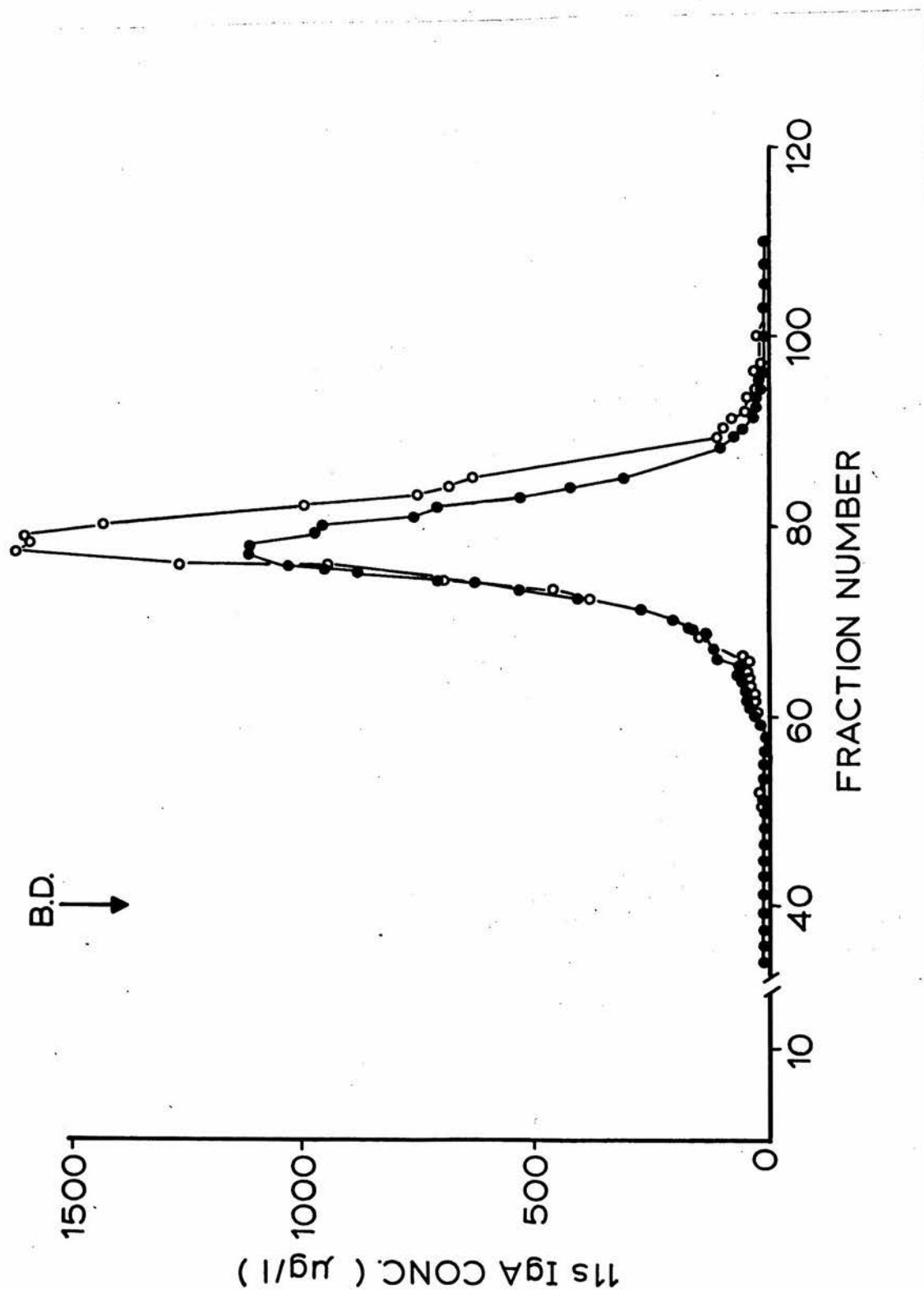


Fig. 4.8: Gel filtration on a Sepharose 4B column of colostrum (o-o-o) and milk (●-●-●). 3 ml fractions were collected and immunoassayed for IgA. The arrow indicates the elution volume of a Blue Dextran marker (B.D.; void volume).

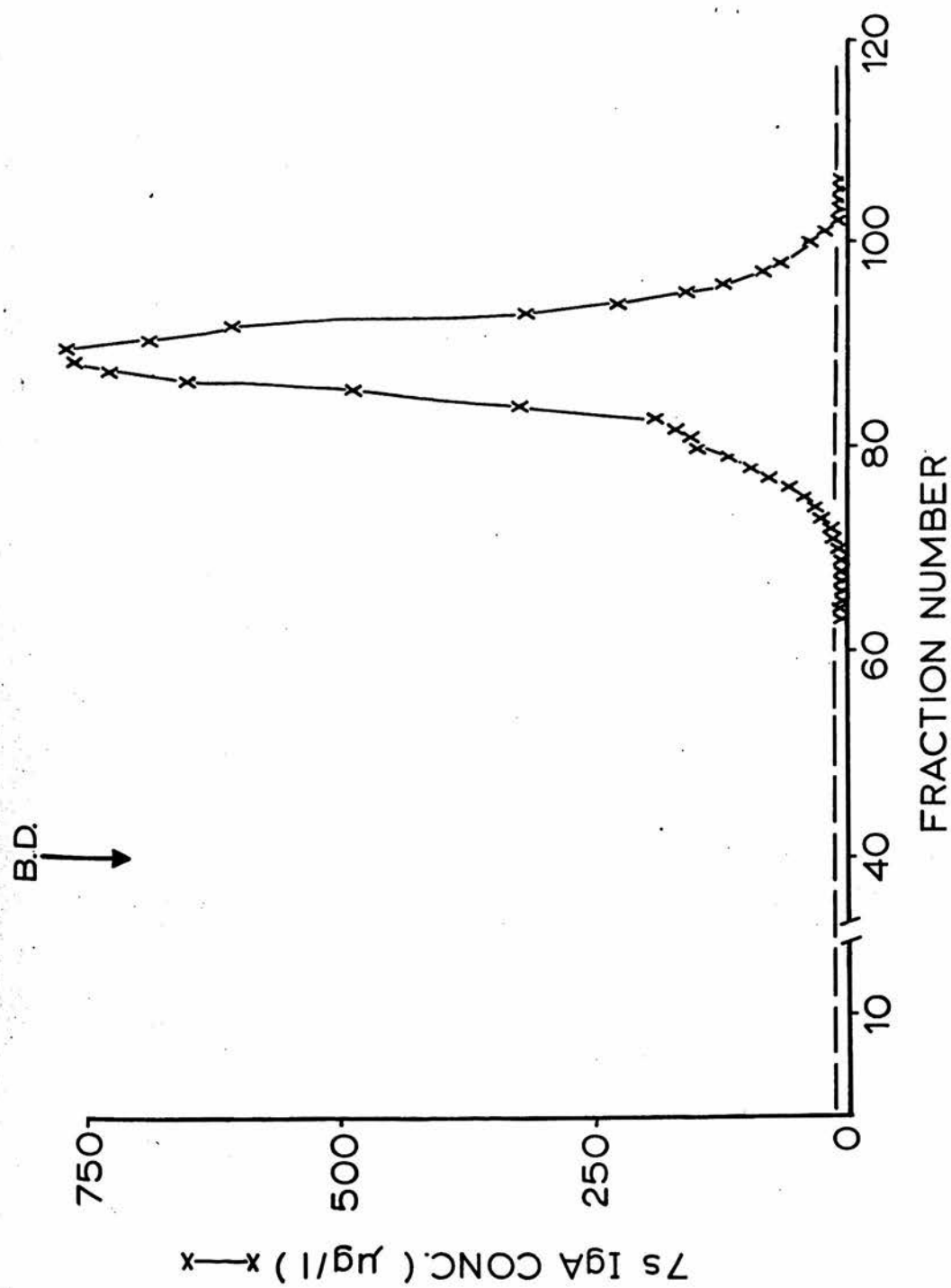


Fig. 4.9:
Gel filtration on a Sepharose 4B column of normal adult serum (x—x) using the same experimental conditions as in Fig. 4.8.

(b) For the determination of 7S IgA using ^{125}I -11S IgA as tracer, the intra-assay CV for samples with mean 7S IgA concentrations of 10.0, 20.5, 42.6, 86.5 and 151.7 ng/ml was 10.9, 11.5, 8.2, 6.3 and 5.5% respectively. The inter-assay CV for samples with mean 7S IgA concentrations of 5.3, 10.2, 20.2, 42.2, 87.5 and 156.7 ng/ml was 21.6, 14.6, 11.2, 11.6, 12.4 and 10.1% respectively.

(c) For the determination of 7S IgA using ^{125}I -7S IgA as tracer, the intra-assay CV for samples with mean 7S IgA concentrations of 5.9, 11.1, 19.3, 40.1 and 90.9 ng/ml was 19.2, 8.2, 7.8, 6.8 and 9.0% respectively. The inter-assay CV for samples with mean 7S IgA concentrations of 4.9, 10.0, 19.3, 41.6 and 88.5 ng/ml was 19.5, 14.9, 9.1, 9.1 and 18.5% respectively.

(d) For the determination of IgG by RIA, the intra-assay CV for samples with mean IgG concentrations of 11.6, 21.5, 42.7 and 82.9 ng/ml was 7.0, 7.4, 5.7 and 6.1% respectively. The inter-assay CV for samples with mean IgG concentrations of 9.5, 18.6, 38.8, 79.0 and 161.9 ng/ml was 18.8, 13.0, 9.4, 10.1 and 8.9% respectively.

4.4 DISCUSSION

In this chapter, radioimmunoassays for 11S IgA, 7S IgA and IgG are described together with an antiserum anti-11S IgA/FSC for detecting the conformational determinant of 11S IgA and the accessible determinant of free secretory component. When considering the measurement of the different forms of IgA, a description of the antigenic determinants and the in vivo synthesis of 11S IgA is necessary.

In serum, IgA is present in the monomeric, 7S form possessing light and heavy α -chain antigenic determinants. This IgA originates in spleen, lymph nodes and bone marrow in addition to gut (Hijmans, Schuit and Hulsing-Hesselink, 1971; Radl, Schuit, Mestecky and

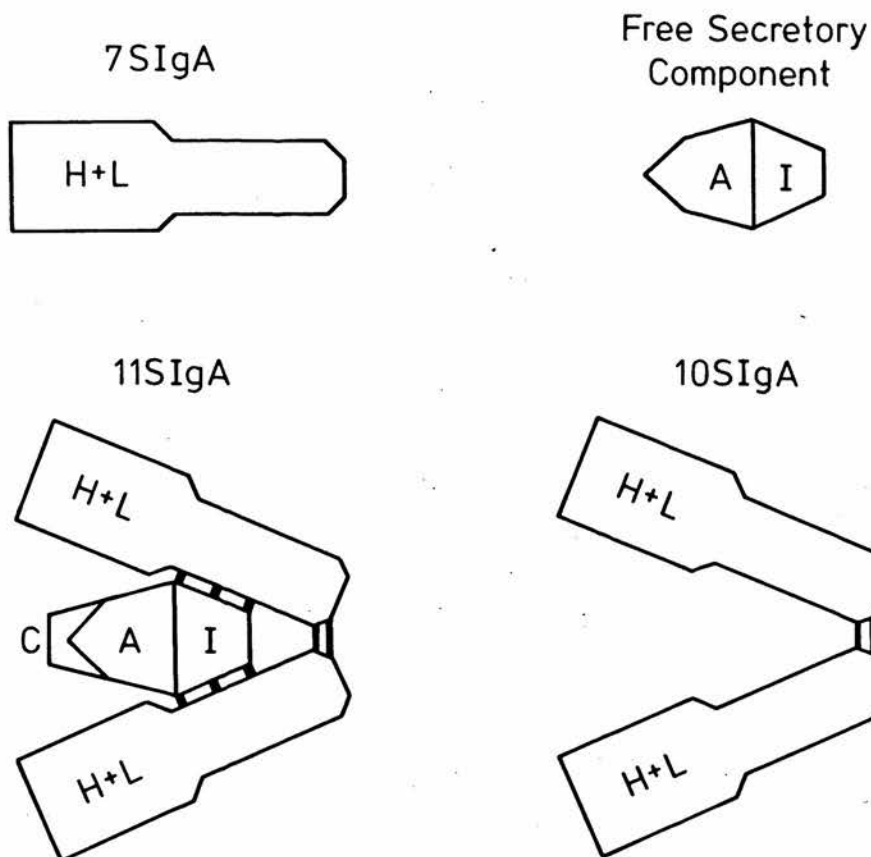
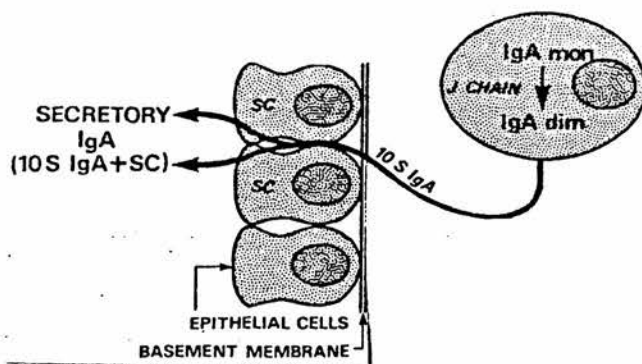


Fig. 4.10:

The synthesis and transport of 11S IgA.

Above: IgA monomer (IgA mon) is converted into the IgA dimer (IgA dim) with the addition of J chain in the sub-epithelial IgA plasma cells; 10S IgA is secreted and converted into 11S IgA by the addition of secretory component (SC) in the epithelial cell, and then secreted into the lumen (from Hauptman and Tomasi, 1978).

Below: Antigenic determinants present on the different forms of IgA and on free secretory component (H, L: heavy and light chain determinants; A, I and C: accessible, inaccessible and conformational determinants respectively).

Hijmans, 1974). In contrast, in external secretions, IgA is present as 11S IgA which is synthesised by epithelial cells from 10S IgA and secretory component (Lamm, 1976). 10S IgA is synthesized by sub-epithelial plasma cells and this synthetic pathway, and the difference in antigenic structure between the different forms of IgA is shown in Fig. 4.10. Secretory component can exist in the free form, with two antigenic determinants: an accessible determinant ('A') and an inaccessible ('I') one. However, when secretory component combines with 10S IgA to form 11S IgA, one of the determinants (I) becomes inaccessible (Brandtzaeg, 1971). In addition, this combination with 10S IgA results in the appearance of a new antigenic determinant, the configurational or conformational determinant ('C') a characteristic of bound secretory component (Brandtzaeg, Fjellanger and Gjeruldsen, 1970).

Thus, when all the different antigenic determinants are considered (Fig. 4.10), it is clear that considerable cross-reaction exists between 7S, 10S and 11S IgA. The following procedure was therefore adopted in many of the studies described in this thesis for the measurement of IgA. The experimental sample was first analysed by constructing binding inhibition curves to assess if the B/B₀ curve for the experimental sample was parallel to the appropriate immunoglobulin standard. Then an aliquot of the sample was analysed by Sepharose 4B gel filtration (or latterly by sucrose density gradient ultracentrifugation). The elution pattern from gel filtration (or density profile from ultracentrifugation) indicated if predominant IgA immunoreactivity was in the 400,000 dalton (10-11S) or in the 150,000 dalton (7S) position. If the IgA immunoreactivity was in the 400,000 dalton position, then gel filtration fractions, and other

experimental samples of the same type were analysed by RIA using ^{125}I -11S IgA tracer, an 11S IgA standard, and anti-7S IgA antiserum. If the predominant immunoreactivity was in the 150,000 dalton position, then RIA using ^{125}I -11S IgA tracer but a 7S IgA standard, and anti-7S IgA antiserum were used instead.

In addition, in some analyses of gel filtration fractions or ultracentrifugation fractions, the peak of immunoreactivity in the 400,000 dalton position was assayed using the antiserum (anti-11S IgA/FSC) that had been adsorbed with serum IgA. With reference to Fig. 4.10, it can be seen that antibody activity was originally present against heavy α -chain, light chain, A and C antigenic determinants. Adsorption with 7S IgA (present in large quantities in the myeloma serum) removed antibody activity against heavy α -chain and light chain determinants. Thus, this antiserum, anti-11S IgA/FSC, no longer reacted with 10S IgA and was used to ensure that the peaks of immunoreactivity detected in the 400,000 dalton (or 10-11S) position were solely due to the complete, native 11S IgA molecule, and not a 10S IgA molecule. It should be noted that in RIA using anti-11S IgA/FSC (with an 11S IgA standard and ^{125}I -11S IgA tracer), the presence of free secretory component would also be detected, since this molecule would also compete with ^{125}I -11S IgA tracer for binding by anti-11S IgA/FSC. Unfortunately, because of the lack of a purified standard for free secretory component, no quantitation of this protein (seen for instance in Fig. 6.4) was possible. If an immunoadsorbent consisting of only free secretory component had been available, an antiserum specific for 11S IgA, reacting only with the conformational determinant could have been prepared and would have been of great value in the study of external secretions.

The inhibition of binding of ^{125}I -11S IgA by the 11S IgA standard was not parallel with the inhibition of binding by the 7S IgA standard, reflecting differences in affinity between the 11S and 7S IgA molecule for the anti-7S IgA antibody, and emphasising the need for the correct IgA standard when analysing experimental samples.

The inhibition of the binding of ^{125}I -11S IgA caused by purified IgG in an 11S IgA RIA was very small but the inhibition of binding of ^{125}I -IgG caused by purified 11S IgA in an IgG RIA was higher. 183 ng of 11S IgA was equivalent to 1ng IgG but it was not clear whether this was due to incomplete adsorption of the rabbit anti-IgG or contamination of the purified 11S IgA preparation with IgG. It was not possible to investigate this problem further due to the difficulty of obtaining preparations of IgA free of IgG impurities.

The reproducibility of the assay was satisfactory except for samples with values at the 'lower' end (i.e. B/Bo greater than 65%) of the standard curve. This was avoided in analysis of experimental samples where possible by assaying the sample at a dilution which would result in a B/Bo value of 40-50%. The limit of detection was not routinely determined since it was found to correspond to the immunoglobulin concentration resulting in 10% binding inhibition in preliminary experiments.

The comparison of RIA using ^{125}I -11S IgA tracer, a 7S IgA standard and anti-7S IgA antiserum with RIA using ^{125}I -7S IgA tracer and the same standard and antiserum demonstrated that a good correlation between the two methods existed when 7S IgA in serum was being measured, thus validating the procedure. Although the use of ^{125}I -11S IgA tracer resulted in a slightly higher value than when ^{125}I -7S IgA tracer was used, the value obtained with the former tracer was not significantly different and correlated well with the value

obtained by SRID or nephelometry confirming the validity of the method.

Similarly, a good correlation existed between 11S IgA RIA and SRID with no significant difference between the values obtained for individual ^{milk samples} / by the two different methods. A good correlation was also obtained between RIA, SRID and nephelometry for the measurement of IgG in serum. However, a significant difference existed for the IgG values for individual sera when RIA was compared with SRID and nephelometry. The reason for this was not clear but may have been due to some factor related to IgG heterogeneity (e.g. subclass composition) affecting RIA differently from SRID and nephelometry.

CHAPTER 5

IgA SYNTHESIS IN VITRO BY NORMAL HUMAN BREAST TISSUE

5.1 INTRODUCTION

The high concentrations of 11S IgA present in colostrum and milk (Hanson, 1961; McClelland et al., 1978) are the result of local synthesis by sub-epithelial plasma cells in the mammary gland (Lamm, 1976). These plasma cells, many of which stain for IgA, are most numerous in the lactating breast (Pumphrey, 1977) and originate from gut-associated lymphoid tissues, consisting mainly of Peyer's patches (for references, see Chapter 1). Some plasma cells staining for IgA are also present in the non-lactating breast (Drife et al., 1976) and are presumably responsible for the high IgA concentrations detected in breast secretions from non-lactating women (see Section 6.4).

The studies described in this chapter were stimulated by the demonstration by Drife et al. (1976) that ^{14}C -amino acid precursors could be incorporated in vitro into IgA by normal breast tissue obtained from non-lactating women. A qualitative difference in ^{14}C -amino-acid incorporation was found between the two phases of the menstrual cycle in parous but not nulliparous women. This finding was interpreted as suggesting that a change in breast sensitivity to progesterone had occurred following the first pregnancy, affecting IgA synthesis. A further investigation of this finding was therefore made by attempting to quantify the observations of Drife et al. (1976), using the IgA RIA. The ability of the RIA to detect IgA secreted by lymphocytes or plasma cells was first tested on peripheral blood lymphocytes. Mammary tissue fragments cultured in roller tubes (Van Furth, Schuit and Hijmans, 1966), or on metal grids (Baker and Neal, 1973) were then studied.

5.2 MATERIALS AND METHODS

Subjects

Peripheral venous blood was obtained from three healthy male volunteers aged 24-34 years; peripheral blood lymphocytes were purified from the blood using Triosil (Nygard)/Ficoll (Pharmacia) gradients and washed thrice with Ham's F10 medium (Flow Labs.) containing 2.5% foetal calf serum (Flow Labs.) prior to culture.

Normal mammary tissue from seven female patients aged 23-36 years were studied by roller tube culture. They had been admitted to hospital for reduction mammoplasty (patient 1) or diagnostic biopsy of a breast lump (patients 2 to 7) that was subsequently found to be benign. In patient 1, mammary tissue was obtained from different areas of the breast, and in patient 7, mammary tissue was obtained from opposite ends of the biopsy specimen. The biopsy technique was similar to that described by Drife et al. (1976).

Normal mammary tissue from three additional female patients aged 19-35 years who had been admitted to hospital for reduction mammoplasty was also studied by the organ culture technique of Baker and Neal (1973).

Lymphocyte culture

Peripheral blood lymphocytes were cultured in Hams F10 medium and 10% foetal calf serum by the method of Waldmann, Durm, Broder, Blackmann, Blaese and Strober (1974). Duplicate cultures of 1 ml volume containing 2×10^6 lymphocytes and 0%, 0.5%, 1.0% and 2.0% Pokeweed mitogen (v/v; Gibco) were incubated for 1, 2, 3, 4, 5, 6 and 7 days at 37°C in air. Culture supernatants were then removed, stored at -20°C till analysis for IgA by RIA using a 7S IgA standard.

Roller tube culture and demonstration of protein synthesis in vitro

The method used was that of van Furth, Schuit and Hijman (1966). Briefly, the method was as follows: tissue specimens were placed in a sterile Petri dish containing Hank's solution, and finely chopped with scalpels; after rinsing, tissue fragments were randomly divided into two separate roller tubes (so that each roller tube contained 60-150 mg wet weight of tissue) and either immediately frozen to -20°C to arrest IgA synthesis, or incubated for 48 hr in 1 ml modified Eagle's medium (Flow Laboratories) containing 1 $\mu\text{Ci/ml}$ ^{14}C -L-lysine (specific activity 342 Ci/mol) and 1 $\mu\text{Ci/ml}$ ^{14}C -L-isoleucine (specific activity 348 Ci/mol; Radiochemical Centre, Amersham), gentamicin (25 $\mu\text{g/ml}$) and nystatin (75 units/ml). After incubation, the tissue fragments that had been either stored at -20°C without culture, or cultured 48 hours, were frozen and thawed successively five times to disintegrate and extract the IgA present in the tissue fragments. The culture supernatants were then removed, and the tissue fragments washed twice with 1 ml buffer; the supernatants and washings for each respective culture were then pooled and an aliquot withdrawn for measurement of IgA concentrations by RIA using an 11S IgA standard. The remainder was dialysed against phosphate buffer (0.15 mol/l, pH 7.6) to remove the excess radioactive amino acids. Next the culture fluid was concentrated by lyophilization and dissolved in 0.1 ml of distilled water.

The culture fluids were analysed by micro-immunoelectrophoresis. Because the concentrated fluids contained insufficient protein to produce a well-defined precipitation line, carriers were used (Lai A Fat, McClelland and Van Furth, 1976). The newly synthesized proteins were detected and identified by autoradiography of the immunoelectrophoretic pattern or Ouchterlony plate. Plates were exposed to Kodak Royal X Pan film (ASA 1200) for 21 days and developed with 10% Rodinal solution.

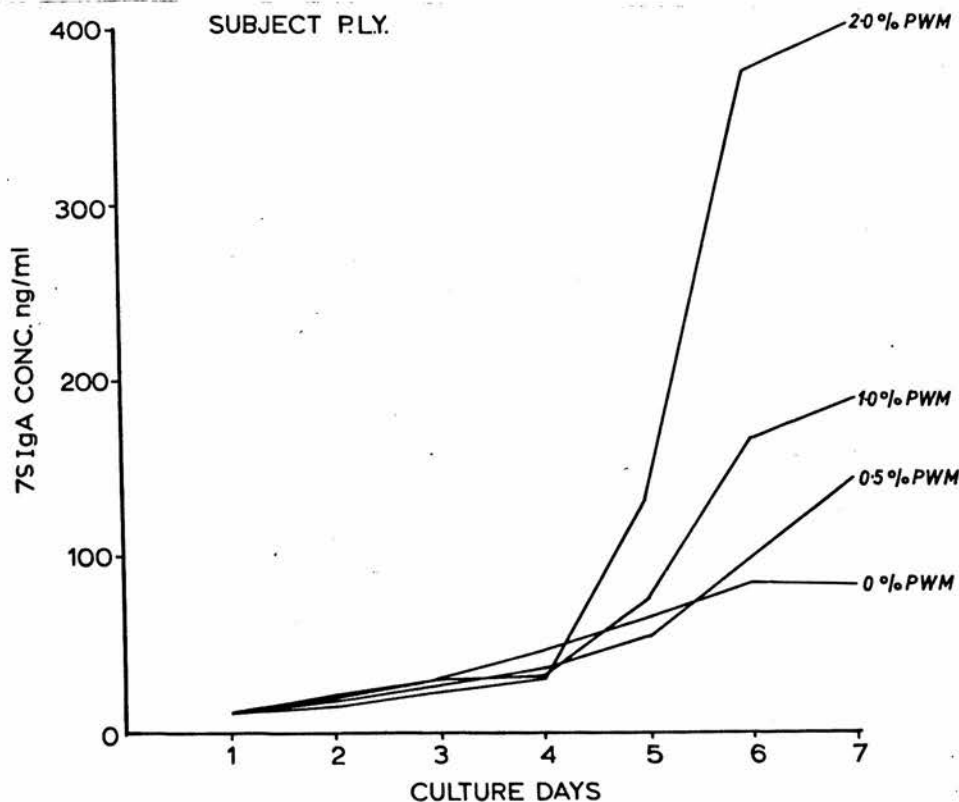
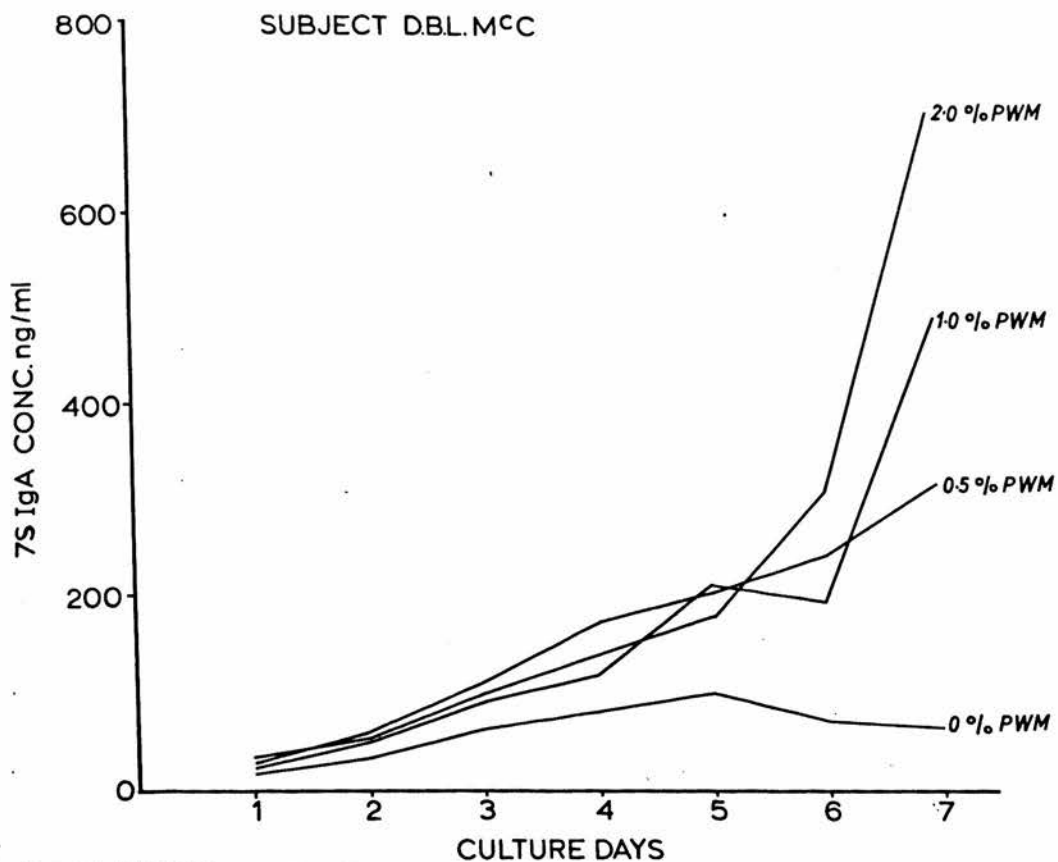


Fig. 5.1:

IgA synthesis by 2×10^6 lymphocytes in vitro.

Subjects 1 (D.B.L. McC) and 2 (P.L.Y.) responded to 0.5%, 1.0% and 2% of Pokeweed mitogen (PWM) with increased IgA synthesis but subject 3 (R.S.; overleaf) by contrast did not show a similar response.

Protein synthesis occurring in vitro was evaluated from the intensity of the respective autoradiographic lines, which indicates the amount of protein synthesised (Van Furth et al., 1966). The intensity was classified according to a scale ranging from - = negative; ⁺ = just visible; + = clearly visible, to a maximum of ++++.

Organ culture by the method of Baker and Neal (1973)

The biopsy material was treated in the same way as described above, but instead of being placed in roller tubes, tissue fragments were incubated for 24, 48 or 96 hours on moist filter paper (Whatman) raised by metal grids just above the level of the culture medium (HEPES buffered Eagle's medium containing 20% newborn calf serum; Flow labs) in an atmosphere consisting of 5% CO₂ in 95% air (10 lb/sq. in.) at 37°C. Some cultures contained glucose (5 mg/ml) or insulin (up to 200 ug/ml; Sigma) in an attempt to improve tissue survival. Other cultures contained colcemid (8 ug/culture; from Dr. T.G. Baker) added 8 hours prior to the termination of the culture, to assess the number of mitoses occurring in culture. Culture media were changed regularly at 24 hour intervals.

At the end of the culture period, tissue fragments were fixed in Bouin's fluid, and processed by standard dehydration and embedding procedures. Serial sections cut at 5 um were stained with haematoxylin and eosin.

5.3 RESULTS

Lymphocyte culture

In all three subjects studied, increasing IgA concentrations of similar magnitude were observed in culture supernatants from the first to the fifth or sixth day of culture, reaching a value of

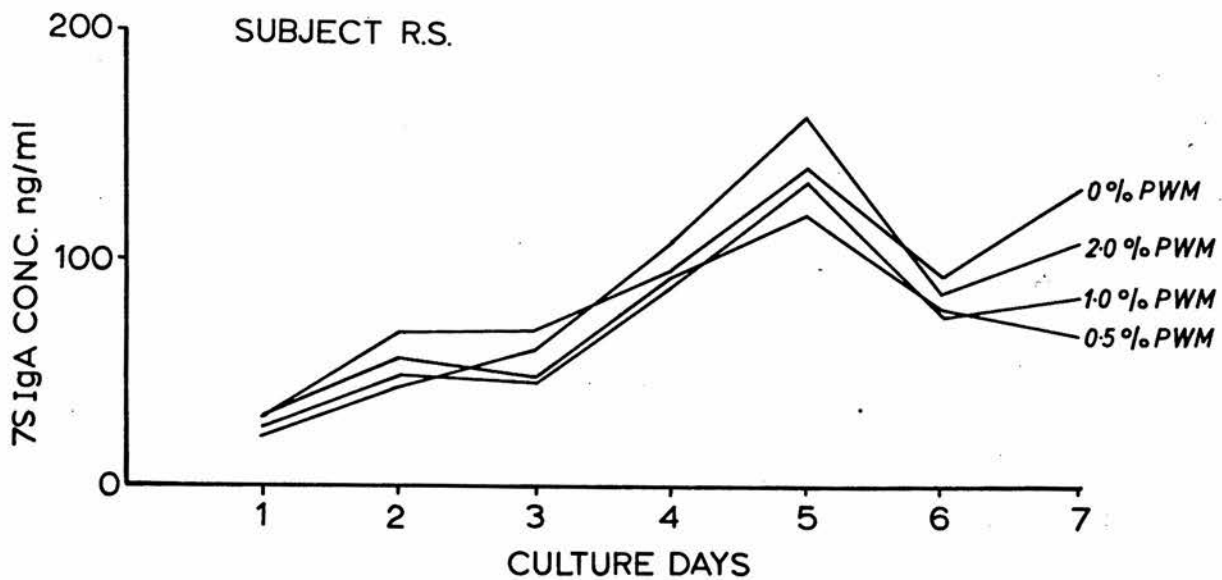


Fig. 5.1 (cont.):

IgA synthesis by 2×10^6 lymphocytes in vitro: subject 3.

approximately 100 ng/ml. This suggested that the net IgA secretion from 2×10^6 peripheral blood lymphocytes in 1 ml was 20 ng of IgA per day.

When the lymphocyte cultures were stimulated with varying doses of Pokeweed mitogen, large increments of IgA secretion were seen in subjects no 1 and 2, but not in subject no 3 after the fifth day of culture, presumably due to suppressor T cell activity (Fig. 5.1).

Roller Tube Culture: Correlation of autoradiography with RIA in the measurement of IgA synthesis

In 11 culture experiments using normal breast tissue from seven patients, IgA synthesis measured qualitatively by autoradiography ranged from - to ++++ and did not correlate with the weight of tissue cultured.

Measurement of IgA concentrations by RIA in the 3 ml pool of culture supernatants and washings permitted a calculation to be made of the total extractable IgA from the tissue fragments in each culture tube. As the weight of tissue fragments was different in each roller tube, the amount of IgA extracted per mg wet weight of mammary tissue was calculated. IgA concentrations ranged from 48-600 ng/mg wet weight in the mammary tissue fragments that had been frozen immediately to arrest IgA synthesis, suggesting that the normal human breast contained substantial amounts of IgA normally or that some contamination with IgA from blood had occurred. IgA concentrations in the mammary tissue fragments that had been cultured 48 hours were similarly calculated, and it was found that only in four cultures did IgA concentrations (on a ng/mg wet weight basis) in tissue fragments cultured 48 hours exceed IgA concentrations in tissue fragments originating from the same biopsy specimen, but frozen immediately (Table 5-1).

Table 5-1

Patient no.	Age	Parity	Culture no.	IgA extractable from tissue fragments frozen immediately (ng/mg tissue weight)	IgA extractable from tissue fragments cultured 48 hr (ng/mg tissue weight)	IgA increment	IgA synthesis assessed by autoradiography
1	29	Parous	610	144.0	153.9	9.9	++
			611	116.4	76.2	0	+
			612	228.5	209.4	0	-
			613	165.7	107.9	0	±
2	31	Parous	618	248.9	264.1	15.2	++++
3	28	Parous	619	254.2	230.3	0	+++
4	36	Parous	620	467.5	509.1	41.6	+++
5	28	Nulliparous	621	47.8	155.2	107.4	±
6	29	Parous	622	109.7	76.9	0	+
7	23	Nulliparous	623	467.0	340.3	0	++
			624	599.6	596.1	0	+++

In one patient (no 1), multiple biopsy specimens were analysed, and it was found that IgA concentrations were similar in biopsy specimens excised from different parts of the breast (Table 5-1).

When the degree of IgA synthesis detected by autoradiography was compared with the 'increment' in IgA concentrations between tissue fragments cultured 48 hours over the corresponding tissue fragments (from the same biopsy specimen) frozen immediately, no correlation was seen (Table 5-1).

Organ culture by the method of Baker and Neal (1973)

Three specimens of normal breast tissue were studied in an attempt to obtain a long term (up to six days) culture system. In an assessment undertaken with Dr. T.G. Baker, it was found that the microscopic appearance of the tissue fragments in haematoxylin and eosin stained sections was only satisfactory in mammary tissue cultured for 48 hr. In tissue fragments that had been cultured 96 or 144 hrs, pyknotic cells were present, and there was a breakdown in the orderly appearance of the ductal and alveolar epithelium. In addition, increasing cell debris was observed in the lumen of the ducts.

The mitotic index (number of cells in division per 1000 cells counted) was generally low in explants cultured for 48 hours and was not increased in an experiment where insulin concentrations in the culture medium were 0, 1, 2, 5, 10 or 50 ug/ml, and glucose concentrations were either 0 or 5 mg/ml. Tissue fragments were found to vary in their content of glandular or ductal tissue and some tissue fragments did not contain any epithelial tissue at all, when serial sections were studied.

5.4 DISCUSSION

The studies in this chapter have demonstrated that it is not possible to measure IgA synthesis by using RIA to measure the amount of IgA extractable from tissue fragments cultured for 48 hours, and comparing it with the amount of IgA extractable from fragments that have not been cultured. Substantial quantities of IgA could be extracted from fragments frozen immediately, and is in accord with the finding by Drife et al. (1976) of IgA deposits in the lumina of ductules in 88% of specimens of normal breast tissue. Alternatively, some contamination with IgA originating from blood could have occurred at the time of biopsy.

The RIA itself could detect as little as 10 ng synthesised by 2×10^6 peripheral blood lymphocytes (PBL) in 24 hours. About 15% of PBL are B (antibody secreting) lymphocytes, and 4% of PBL are class-specific for IgA (Ammann, Borg, Kondo and Wara, 1977). This suggests that about 80,000 PBL are capable of secreting 10 ng of IgA per 24 hours. In the normal, non-lactating breast, a calculation can be made for the number of plasma cells present, most of which will stain for IgA (Drife et al., 1976) based upon a mean value of 2.72 plasma cells per lobule ($n = 40$, J.O. Drife, personal communication) and a lobular density of 67 lobules per cm^2 in the third decade of life (Wilson, 1976). If one assumes that no shrinkage of the specimen occurs on fixation and processing of the tissue, and that sections of 5 μ are made with a plasma cell appearing in only one section, then the number of plasma cells in cube of breast tissue measuring 1 cm x 1 cm x 1 cm will be $2.72 \times 67 \times 2 \times 10^3 = 3.6 \times 10^5$. If it is further assumed that a 1 cm^3 of breast tissue weighs 1 gm, then a 200 mg fragment of breast tissue will contain about 0.7×10^5 plasma cells.

Although PBL differ from plasma cells in the mammary gland in their state of differentiation, organ of origin (Lamm, 1976), control of IgA synthesis (Elson, Heck and Strober, 1979), type of IgA synthesised (Lamm, 1976) and possibly in their rate of synthesis, nevertheless, as a crude estimation, it can be calculated that the plasma cells present in the tissue fragments in a roller tube may secrete 10 ng of IgA (or a value similar in order of magnitude) in 24 hr. Because of the high IgA concentration in the tissue fragments prior to culture (mean value = 250 ng/mg tissue weight), it is unlikely that synthesis of IgA would be detected, without an improved method of removing IgA already present in the biopsy, but trapped in the ductules. The failure to detect an increment of IgA during a 48 hr. culture period is therefore in accord with the calculation based on PBL IgA secretion rate.

Long term organ culture on metal grids was attempted because this method is mechanically less damaging than roller tube culture (D.B.L. McClelland, personal communication). The method of Baker and Neal (1973) has been used successfully for organ culture for up to 16 days (O and Baker, 1976) but it was found that mammary tissue fragments could only be cultured successfully for 48 hr. The failure to culture mammary tissue for longer periods may have been due to the wrong choice of culture medium or additives, as other workers have managed to culture normal breast tissue for longer periods (Barker, Fanger and Farnes, 1964; Ceriani, Contesso and Nataf, 1972; Flaxman, 1974; Dilley and Kister, 1975) but it was felt that a long culture period would be required for ^{the} / synthesis of IgA, sufficient to be distinguished from the IgA present in tissue fragments that had not

been cultured, and from the experimental variation in the tissue fragments themselves due to the histological heterogeneity of the breast (Short and Drife, 1977). No further experiments were therefore undertaken to optimize culture conditions or to study IgA synthesis in vitro using RIA.

CHAPTER 6

STUDIES ON BREAST SECRETIONS

6.1 GENERAL INTRODUCTION

In this chapter, various secretions from the breast were studied with the aim of elucidating the functional state of the secretory immune system within the human breast at periods of life other than post-partum lactation. It is known that lactation sometimes takes place in the neonate, the secretion being known as 'witch's milk'. Lactation also occurs in some women with hyperprolactinaemia due to causes other than pregnancy (galactorrhoea), and secretions from the nipple can sometimes be expressed in response to manual compression (Lauric, 1975) or negative pressure exerted via a suction cup (Sartorius, 1973) in healthy nulliparous or parous women who are not hyperprolactinaemic. Finally, the breast also 'secretes' fluid into cysts in gross cystic disease of the breast. This cyst fluid might be considered to be a 'static' secretion.

The concept of a distinct secretory immune system arose as a result of studies indicating that IgA was the predominant immunoglobulin in several external fluids and originated from sub-epithelial plasma cells (for review, see Lamm, 1976). Secretions may also contain other immunoglobulins, some of which, such as IgM and IgE, may in large part be produced locally. Thus, the concept of the secretory system extends to other immunoglobulins in addition to IgA.

There are two common characteristics for ^{the}majority of external secretions: firstly, the proportion of the different classes of immunoglobulins in secretions varies significantly from the proportions in serum, and secondly, independent regulation of serum and secretory

antibody content occurs, either through local synthesis and/or selective transport. Biologically, these phenomena are of considerable importance since they may lead to a dissociation between systemic and local mucous membrane immunity. Furthermore, these phenomena would suggest that for diseases occurring in the epithelial surfaces of organs which are part of the secretory immune system (e.g. the breast), the appropriate type of immunity to study might be secretory rather than humoral (Roberts, Bathgate and Stevenson, 1975; Pettingale, Merrett and Tee, 1977; Wang, Goodwin, Bulbrook and Hayward, 1977).

The plasma cells in the lactating breast arise from gut-associated lymphoid tissue, as demonstrated by studies of antibodies present in milk (Adinolfi *et al.*, 1966; Allardyce *et al.*, 1974; Goldblum *et al.*, 1975) but the factors that control the migration to, and maturation of IgA precursor lymphocytes within the human breast are unknown.

In this chapter, IgA concentrations were measured in all secretions and fluids studied. In addition, the molecular size and sedimentation coefficient of the IgA detected was measured to confirm that it was 11S. Further confirmation of the molecular form of IgA detected was obtained in some cases, using the antiserum (anti-11S IgA/FSC) specific for the conformational determinant of 11S IgA and the accessible determinant of free secretory component (Brandtzaeg, 1971). This was to ensure that it was not 10S IgA that was being detected, as its occurrence would have indicated a possible abnormality of the secretory immune system.

IgG and albumin concentrations were studied, because the presence of these proteins in external secretions indicates passive

transfer from serum (Brandtzaeg, Fjellanger and Gjeruldsen, 1970). The concentrations of lactoferrin and lysozyme were also studied as these proteins might act as 'markers' of epithelial cell responses to hormonal stimulation (Masson, Heremans, Priguot and Wanters, 1966; Kraus and Mestecky, 1971). IgM concentrations were not studied because concentrations in milk are much lower than the concentrations observed in serum (McClelland et al., 1978) and because there was no obvious way of distinguishing IgM in secretions originating from serum, from IgM originating from local synthesis.

6.2 STUDIES ON NEONATAL MILK

6.2.1 Materials and Methods

Neonatal milk

Five samples of neonatal milk of 0.05 to 0.25 ml volume were collected by manual expression from four neonates (Table 6-1) from 6-28 days post-partum by Dr. Ann Harvie. Additional secretions were obtained from a further nine neonates (whose results are shown in Table 6-2) by Dr. John McKiernen. In one neonate, the neonatal milk was diluted twenty-fold with sodium phosphate buffer to provide a sufficient volume of material for analysis. All samples were stored at -20°C. Blood samples were not obtained as these were healthy infants, other than neonates 6, 7, 8 and 13 who were ill.

Quantitation of IgA

IgA was measured by RIA using an 11S IgA standard.

Quantitation of IgG, lactoferrin, lysozyme and albumin

Single radial immunodiffusion was used to measure the concentration of IgG, lactoferrin, lysozyme and albumin using the reagents

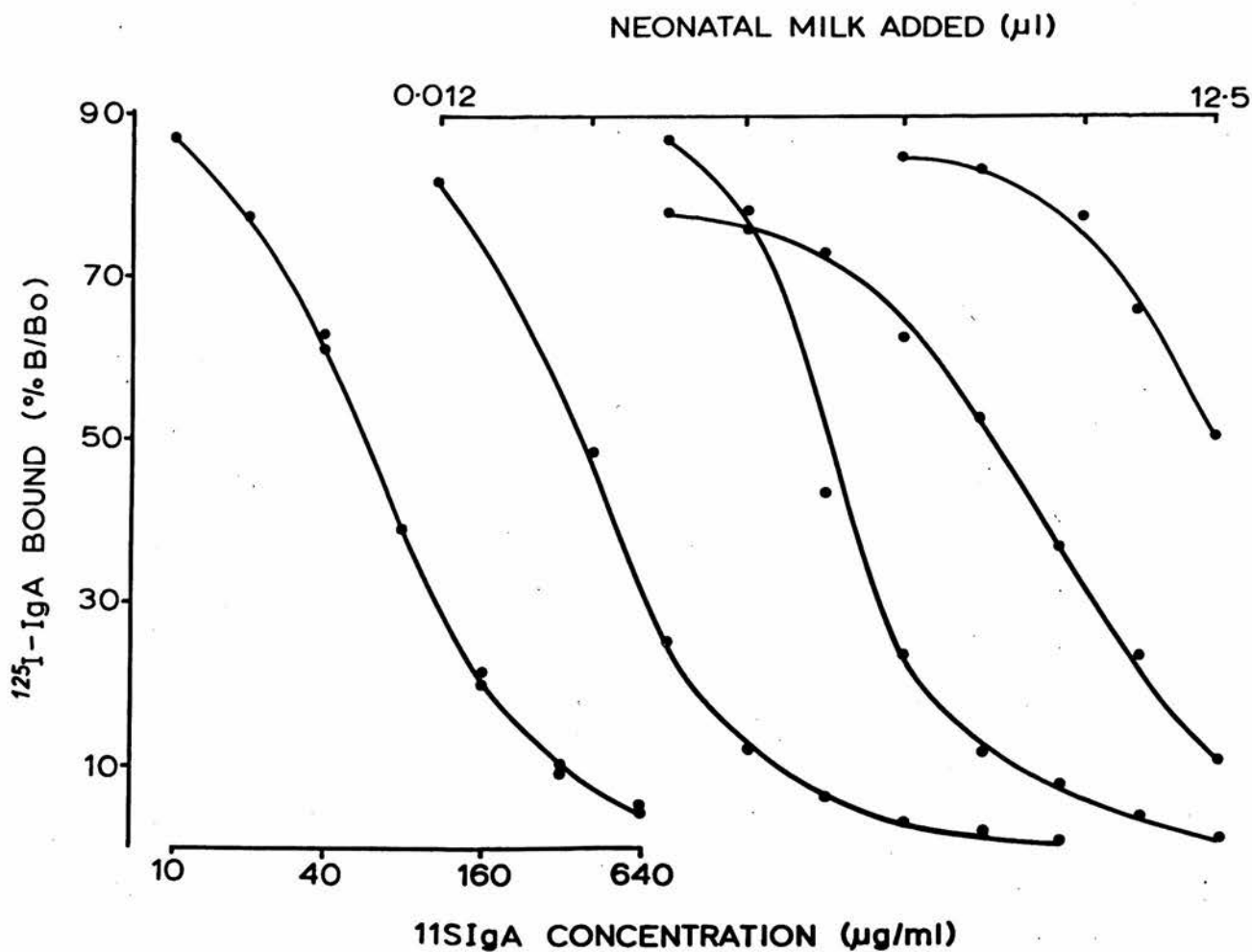


Fig. 6.1:

Binding curves demonstrating parallel inhibition of ^{125}I -11S IgA binding: from left to right, inhibition curve for 11S IgA standard, and inhibition curves for samples of neonatal milk from each of four neonates.

and standards described by McClelland et al. (1978). In neonates 5-13 IgG was measured by RIA instead.

Sedimentation Coefficient Determination

A 25% to 45% sucrose density gradient (236,000 x g for 3 hours, Sorvall OTD-50) was used to determine the sedimentation coefficient of IgA in a sample of neonatal milk. Fractions of 0.1 ml were collected and stored at -20°C prior to immunoassay of the unconcentrated fractions. ^{125}I -7S IgA and ^{125}I -7S IgG were prepared in the same way as ^{125}I -11S IgA and used as markers.

6.2.2 Results

Standard inhibition curve for IgA

The binding of ^{125}I -11S IgA to rabbit anti-7S IgA was displaced in a parallel manner by the 11S IgA standard and three of the four samples of neonatal milk, suggesting that immunoassayable 11S IgA was present (Fig. 6.1). Due to the small volume, it was not possible to plot a complete binding inhibition curve for the remaining sample of neonatal milk.

Sedimentation coefficient determination of IgA in neonatal milk

A single peak of immunoassayable IgA was found on density gradient ultracentrifugation. The peak of immunoassayable IgA was very close to the peak of radioactivity observed for ^{125}I -11S IgA; the difference in position is within the limits of experimental error. In contrast, the peak of IgA immunoreactivity in neonatal milk differed in position by eight fractions from the peak of radioactivity seen for ^{125}I -7S IgA and ^{125}I -7S IgG (Fig. 6.2). It was therefore confirmed that 11S IgA was present in neonatal milk and that 11S IgA was the correct standard to use for quantitation of IgA by double antibody radioimmunoassay (Table 6-1 and 6-2). It was not possible to study

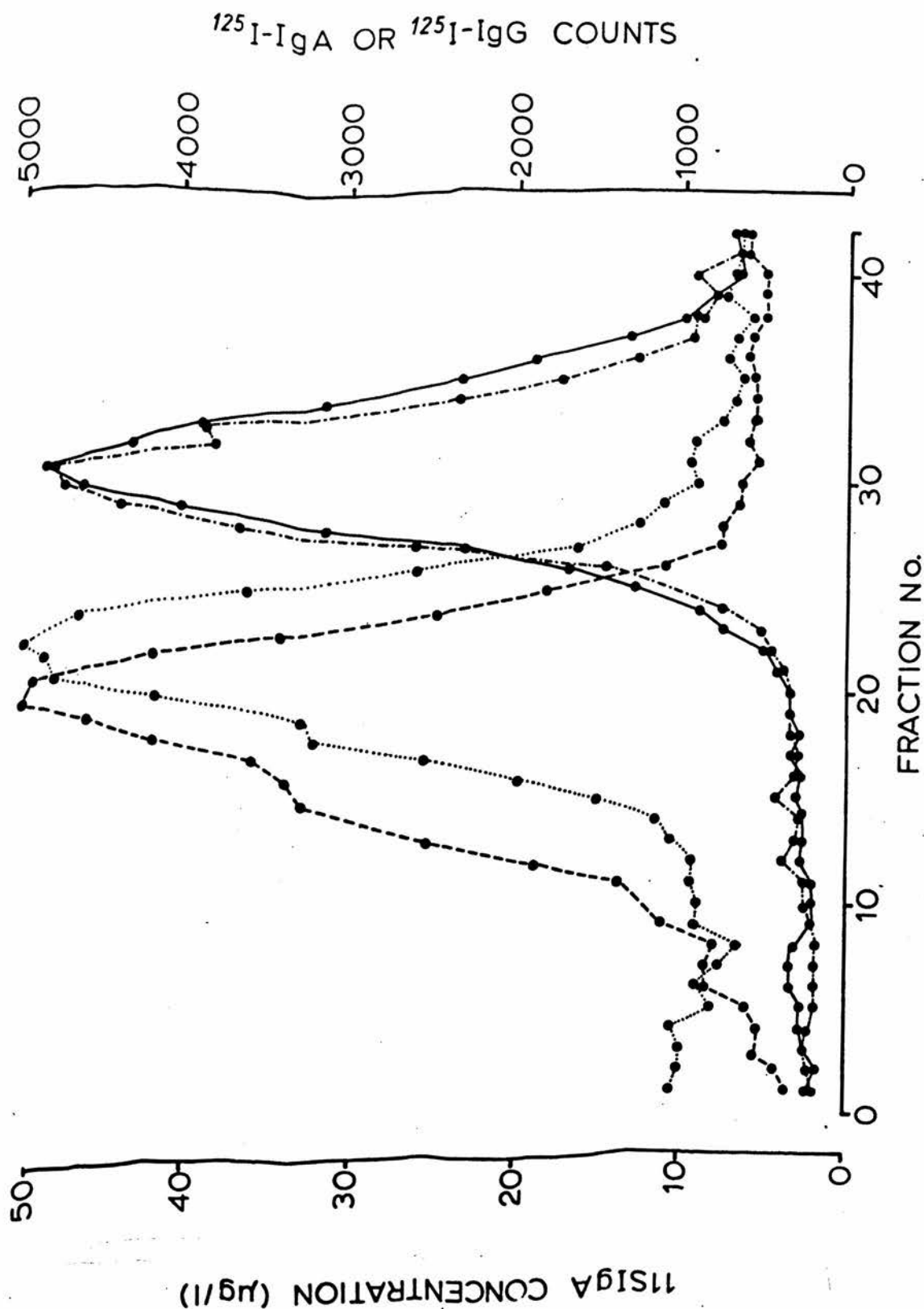


Fig. 6.2:

Sedimentation coefficient determination of a sample of neonatal milk (●.....●) by sucrose density gradient ultracentrifugation. Markers consisting of ¹²⁵I-7S IgA (●—●—●), ¹²⁵I-11S IgA (---●---●---) and ¹²⁵I-7S IgG (-.-.-●-.-.-●-.-.-) were also simultaneously analysed in additional identical sucrose gradients; 0.1 ml fractions were collected and assayed by RIA (neonatal milk) or counted in a γ-counter (¹²⁵I-labelled proteins).

the fractions collected from ultracentrifugation using anti-11S IgA/FSC as described for galactorrhoeic mammary secretions in the next section. This was due to the large peak of immunoreactivity caused by free secretory component obscuring the smaller 11S IgA peak (not shown).

Quantitation of IgG, lactoferrin, lysozyme and albumin

In neonatal milk, IgG, lactoferrin, lysozyme and albumin were detected: the concentrations were similar to those in maternal milk collected on the sixth day post-partum (McClelland et al., 1978). The concentrations of IgG in neonatal secretion were very much lower than serum IgG concentrations at birth (Tables 6-1 and 6-2).

6.2.3 Discussion

The concentrations of proteins present in normal maternal milk were measured in neonatal milk so as to gain insight into the early development of the secretory immune system. The visible mammary hypertrophy with milk-like secretion (sometimes called witch's milk from the historical belief that it was supernatural in origin, Forbes, 1950) have been attributed to hormonal changes in the neonate that parallel the changes which occur in the mother during the immediate post-partum period (Lyons, 1937; Hiba, del Pozo, Genazzani, Pusterla, Lancranjan, Sidiripoulos and Gunti, 1977). As IgA can usually be found in external secretions, before it is found in serum (Haworth and Dilling, 1966; McKay and Thom, 1969), and as IgA is detectable in low concentrations in serum on the sixth day of life (see Chapter 7), it was surprising that an earlier study had indicated that IgA was absent in neonatal milk (Hanson, 1961).

The purpose of this study was to ascertain the concentration and form of IgA present in neonatal milk. In addition, concentrations of lactoferrin and lysozyme were determined as 'markers' of epithelial

Table 6-1: Milk protein concentrations in neonatal milk, neonatal serum
maternal milk and maternal serum

Neonate	Day of Collection	11S IgA (mg/l)	Neonatal Milk IgA Serum IgA	IgG (mg/l)	Neonatal Milk IgG Serum IgG	Lactoferrin (mg/l)	Lysozyme (mg/l)	Albumin (mg/l)	Neonatal Milk Albumin Serum Albumin
1	Day 6	0.43	0.16	100	0.010	7,050	32	330	0.008
	Day 8	0.46	0.18	N.D.		N.D.	N.D.	N.D.	-
2	Day 16	3.25	1.25	20	0.002	11,200	380	360	0.009
3	Day 19	118.17	45.45	N.D.	-	900	N.D.	N.D.	-
4	Day 28	17.57	6.76	80	0.008	7,000	54	290	0.007
Neonatal Serum		2.6 ⁺ 1.45*		10420 ⁺ 592**		N.D.**	N.D.**	40110 ⁺ 1424**	
Maternal*** Milk	Day 6	3,680	2.97	39	0.007	4,950	115	360	0.010
Maternal** Serum		1,239		5690		N.D.	6.0	34860	

* Mean \pm SEM (n = 48) from Chapter 7 of this thesis.

** Mean \pm SEM (n = 19) by McClelland and Samson (unpublished observations on cord blood and maternal blood).

*** Mean values from McClelland et al. (1978)

N.D. Not detected due to dilution of secretion (neonate 3), or due to insufficient secretion for analysis (neonate 1, day 8).

cell responses to hormonal stimulation (Masson et al., 1966; Kraus and Mestecky, 1971). The concentrations of IgG and albumin were also determined as an indication of the transit of blood proteins into milk (Brandtzaeg et al., 1970).

The results of the ^{125}I -11S IgA binding inhibition curves and sucrose density gradient ultracentrifugation indicate that the IgA in neonatal milk is of the 11S type. No 7S IgA was detected in neonatal milk. Furthermore, the ratio of neonatal milk IgA levels to neonatal serum IgA levels is very much higher than the ratio of neonatal milk IgG and albumin levels to serum IgG and albumin levels at birth respectively (McClelland and Samson, unpublished observations on cord blood). This difference is particularly marked for neonates 3 and 4.

This indicates that the presence of 11S IgA in neonatal milk is not due to passive diffusion, and that there must be either selective transport of IgA from serum to milk or local production of 11S IgA. In the analysis by gel filtration of both cord blood and neonatal serum, described in Chapter 7 of this thesis, it was found that less than 2.5% of the IgA present has an elution volume similar to that of 11S IgA. The data in this section therefore provides strong evidence for the local synthesis of 11S IgA in the neonatal mammary gland, probably in a similar manner to that occurring in the maternal mammary gland.

Absolute levels of 11S IgA in neonatal milk are approximately a thousand-fold lower than the levels seen in maternal milk. However, this finding closely parallels the observation that neonatal serum IgA concentrations are also a thousand-fold lower than maternal serum IgA concentrations (Chapter 7, this thesis), and presumably reflects

Table 6-2: Sequential milk protein concentrations in neonatal milk

Neonate	Gestation (weeks)	Day of Collection	11S (mg/l)	IgA (mg/l)	IgG (mg/l)	Lactoferrin (g/l)	Lysozyme (g/l)	Albumin (g/l)
5	40	day 9	0.74	20.3	17	0.1	0.65	
		15	20.40	29.7	IS	IS	IS	
6	41	day 10	0.96	13.6	11	ND	0.20	
		11	2.30	18.7	IS	IS	IS	
		12	3.01	12.5	16.2	ND	0.18	
		13	4.72	10.4	14.0	ND	0.18	
7	40	day 28	341.0	76.0	IS	IS	IS	
		29	576.3	68.1	13.8	0.38	0.83	
		31	608.0	89.3	23.6	0.50	0.83	
8	40	day 9	3.97	28.3	14.0	ND	1.07	
		10	6.69	17.1	8.6	ND	0.62	
9	40	day 14	3.80	55.4	18.0	0.48	0.82	
		28	8.10	43.8	14.0	0.80	0.69	
10	40	day 14	3.94	29.9	14.1	0.14	0.57	
		28	97.96	110.3	26.4	0.17	0.42	
11	40	day 9	1.39	25.9	36.4	ND	0.58	
		13	9.19	15.1	20.8	0.09	0.46	
12	37	day 6	2.67	25.5	13.6	ND	0.29	
		18	403.5	63.2	43.2	0.10	0.57	
13	40	day 4	3.30	28.3	28.0	0.09	0.56	
		6	9.39	23.4	10.2	ND	0.32	

ND = not detected

IS = insufficient secretion for analysis.

Neonates 6, 7, 8 and 13 were ill.

the immaturity of IgA synthesis in the neonate. The presence of relatively high 11S IgA concentrations in the mammary secretions of neonates 12 and 13 indicates that soon after birth, IgA secreting plasma cells are present in the neonatal mammary gland.

In contrast to 11S IgA, the concentrations of lactoferrin and lysozyme are similar to those found in maternal milk. As these two proteins originate in the glandular acini (Masson et al., 1966) and intralobular ducts (Kraus and Mestecky, 1971) respectively, this finding suggests that neonatal mammary epithelium responds to the hormonal changes of pregnancy and the immediate post partum period in the same way as maternal mammary epithelium. It is not known whether the high concentrations of lactoferrin and lysozyme are due to epithelial proliferation, increased synthesis by epithelial cells, or both, but the histological appearance of the mammary gland of a full term foetus consists of swollen glandular acini with extensive epithelium, suggesting that epithelial proliferation is occurring (Dabelow, 1957).

In ten neonates, it was possible to study more than one sample of breast secretion from each neonate, and it is clear from the data in Table 6-2 that 11S IgA concentrations increase with the day of collection post-partum, for each neonate as well as between neonates (e.g. neonate 8 and 13 compared with neonate 7, 10 and 12). IgG, lactoferrin, lysozyme and albumin concentrations do not behave in the same way suggesting that these results are not due to volume changes of secretion, and that it is the age of the neonate that influences the 'maturity' of the mammary secretory immune system. This hypothesis is strengthened by the data for neonate 12, who was born premature, and yet on day 18 post partum (equal to a gestational

age of 40 weeks) had 11S IgA concentrations in neonatal milk far higher than the concentrations observed in neonates 1 and 13 in the first week of life.

In the mouse, it has been suggested that the migration to and maturation within the mammary gland of precursor IgA plasma cells is due to some undefined influence of the mammary epithelium following stimulation by the hormones of pregnancy (Weisz-Carrington, Roux, McWilliams, Phillips-Quagliata and Lamm, 1978). The data on lactoferrin and lysozyme concentrations suggests that the initially low absolute concentrations of IgA in neonatal milk may be due to a relative lack of precursor IgA lymphocytes rather than to a lack of "attraction" by mammary epithelium. However, it is possible that antigenic stimulation of the gut post-partum causes division of IgA precursor lymphocytes in the Peyer's patches, resulting in an influx of lymphocytes into glandular sites throughout the body including the neonatal mammary gland. This would explain the rise in 11S IgA concentrations in neonatal milk with the age of the neonate.

Table 6-3: Clinical Data of Patients Studied

Patient no.	Age (years)	Duration of galactorrhoea	Diagnosis	Serum prolactin (mU/l) *
1	35	16 years	Pituitary tumour	11,900
2	28	9 months	Idiopathic	650
3	23	22 months	Post-partum	2,291
4	27	10 months	Drug-induced	641

* Normal range = < 360 mU/l.

6.3 STUDIES ON GALACTORRHOEIC MAMMARY SECRETIONS

6.3.1 Materials and Methods

Mammary Secretions

Four amenorrhoeic female patients with proven hyperprolactinaemia consented to manual expression of mammary secretions; the secretions (0.2 to 1.0 ml volume) were collected by Drs. A. Brooks, C. Martin and C. Vaughan-Williams and stored at -20°C . The clinical information and serum prolactin concentrations for these patients is tabulated in Table 6-3; all patients were parous except for one (no. 4); in all cases, the prolactin levels were previously determined by a specific double antibody RIA (Hwang, Guyda and Friesen, 1971).

Quantitation of IgA

IgA was measured by a double antibody RIA using an 11S IgA standard and the antiserum (rabbit anti-7S IgA) raised in rabbits immunised with serum IgA (Platts-Mills and Ishizaka, 1975). In addition sucrose fractions from sedimentation coefficient determinations were further analysed using anti-11S IgA/FSC.

Quantitation of IgG, lactoferrin, lysozyme and albumin

IgG, lactoferrin, lysozyme and albumin were determined by single radial immunodiffusion (Fahey and McKelvey, 1965) using reagents and standards described previously (McClelland *et al.*, 1978).

Sedimentation Coefficient Determinations

Sedimentation coefficient determinations were performed as described in the previous section for neonatal milk. However, immunoassay was carried out using either rabbit anti-7S IgA, or rabbit anti-11S IgA/FSC. Two different standards were used: a serum IgA standard that had been standardised against the WHO standard (Rowe *et al.*, 1972) for a sedimentation coefficient determination of the IgA in normal adult serum, and an 11S IgA standard, for IgA sedimentation coefficient

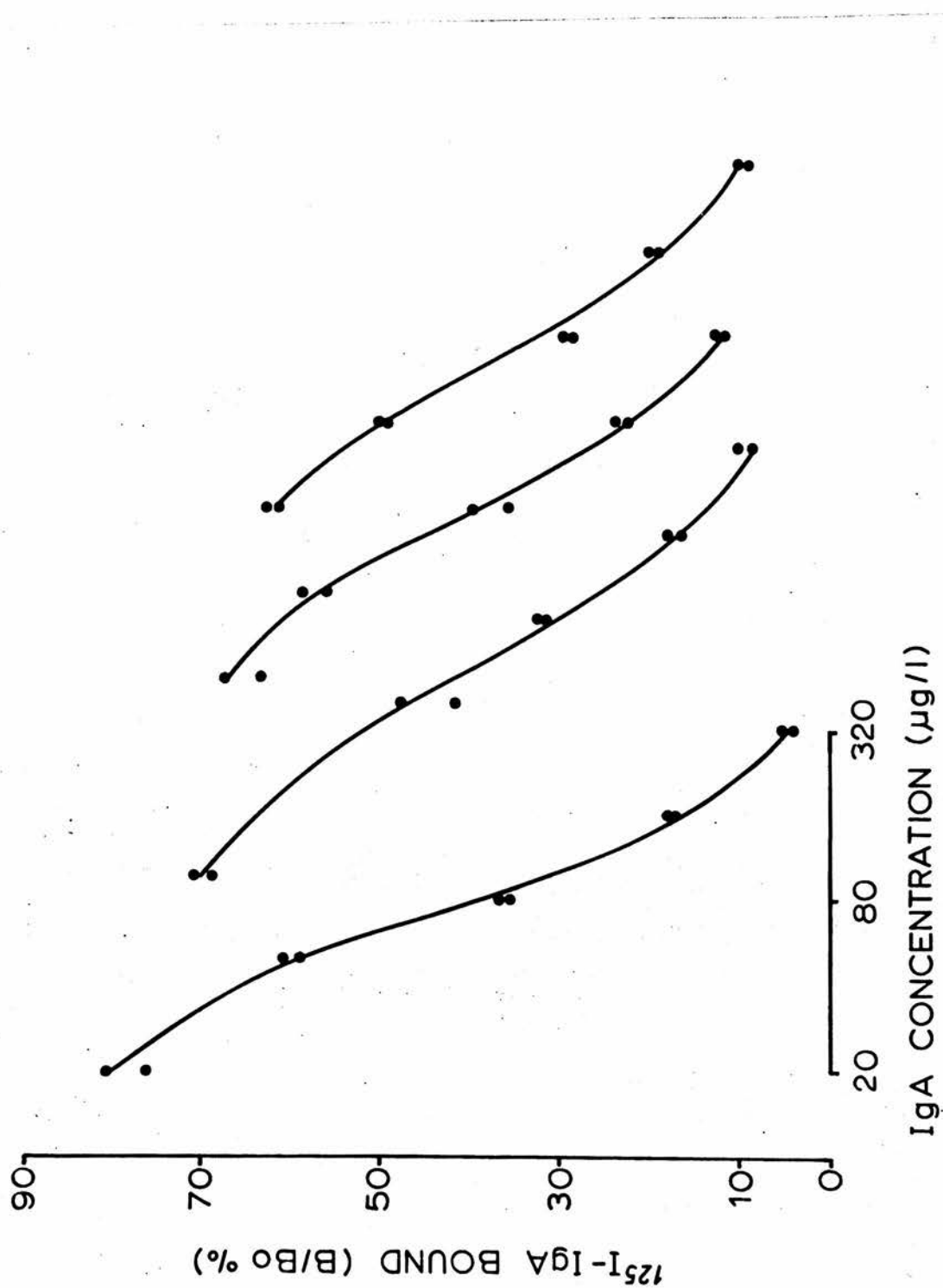


Fig. 6.3: ^{125}I - ^{115}I IgA binding inhibition curves for (from left to right) the ^{115}I IgA standard and samples of galactorrhoeic mammary secretion from each of three patients.

determinations for colostrum, milk and a sample of galactorrhoeic secretion. Three marker proteins were used; two non-radioactive markers consisting of 7S human IgG and a 4.5S Bovine serum albumin marker, prepared by the method of Stanworth, James and Squire (1961) by Mr. B. Freedman, and a radioactive ^{125}I -11S IgA marker prepared as described in Chapter 3.

Gel filtration

Gel filtration was carried out using Sepharose 4B (Pharmacia) in a 2.2 x 90 cm column with downward flow of eluate (Na_2PO_4 , 0.05 mol/l at pH 7.4). Fractions of 3 ml volume were collected and stored unconcentrated at -20°C till immunoassay using rabbit anti-7S IgA.

6.3.2 Results

Standard inhibition curve for IgA

The binding of ^{125}I -11S IgA was displaced in a similar manner by the 11S IgA standard and samples of mammary secretion from each of three galactorrhoeic patients. The binding was not entirely parallel, suggesting that there might be slight antigenic differences between the IgA present in galactorrhoeic secretions and the IgA present in the 11S IgA standard (Fig. 6.3). Galactorrhoeic secretion from the fourth patient was not analysed in this way as it was not available at the beginning of this study.

Sedimentation Coefficient Determination

When the sucrose fractions obtained from ultracentrifugal analysis of colostrum, milk, galactorrhoeic secretion and normal serum were immunoassayed using the rabbit anti-7S IgA, single peaks of immunoreactivity were seen in all instances. The peak of immunoreactivity for the galactorrhoeic secretion studied was identical in position to the peak of immunoreactivity for colostrum,

^{125}I - ^{113}I IgA

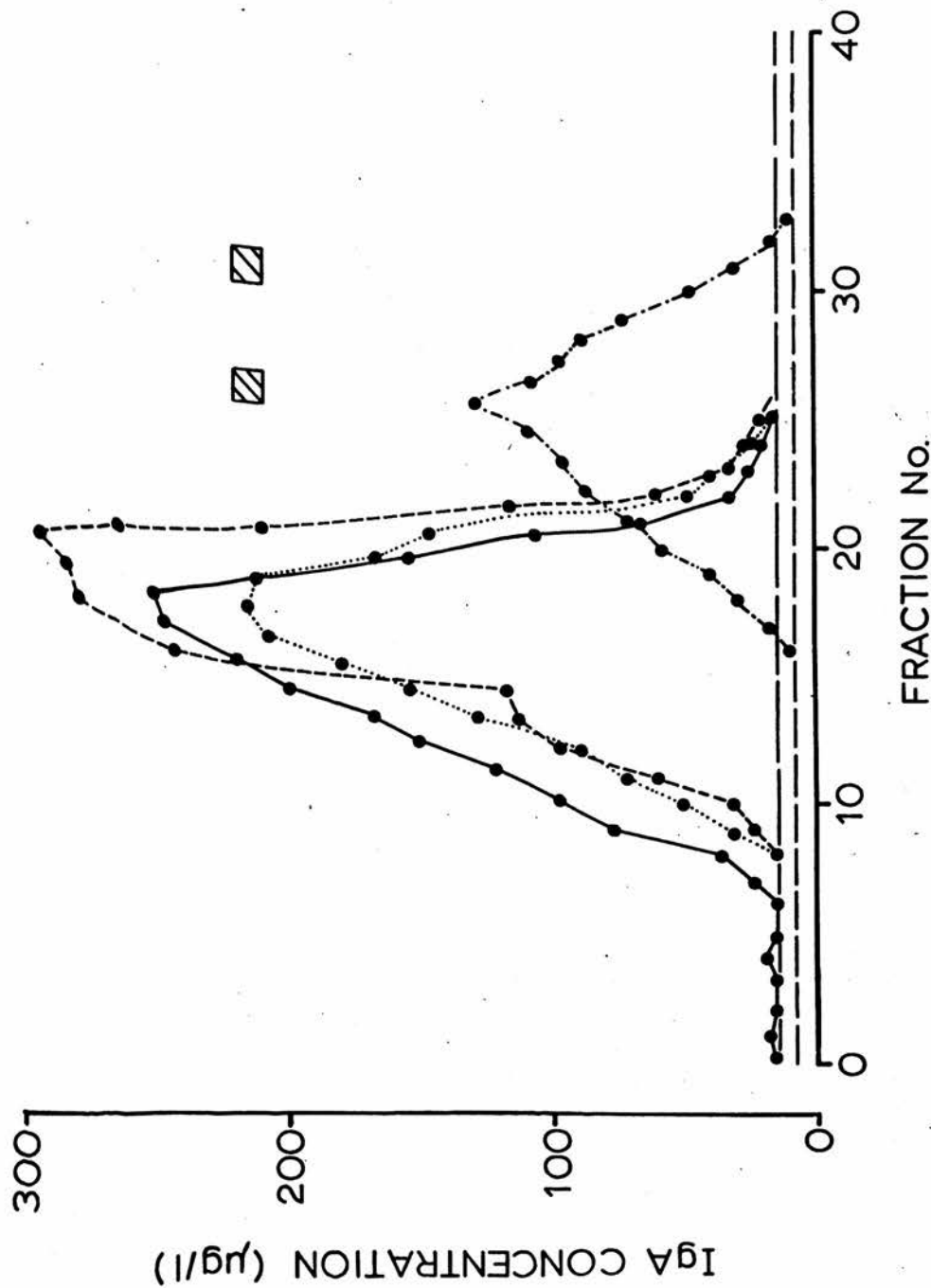


Fig. 6.4:

(a) Sucrose density gradient ultracentrifugation to determine the sedimentation coefficient for colostrum (●—●), milk (●---●), a sample of galactorrhoeic secretion (●.....●) and normal serum (●-.-.-●). A 25% to 45% w/v gradient was used, and 0.1 ml fractions collected from the bottom of the tube and immunoassayed using rabbit anti-7S IgA antiserum and an 11S IgA standard (colostrum, milk and galactorrhoeic secretion) or a 7S IgA standard (serum). The two hatched areas represent (from left to right) the position of the non-radioactive 7S and 4.5S markers.

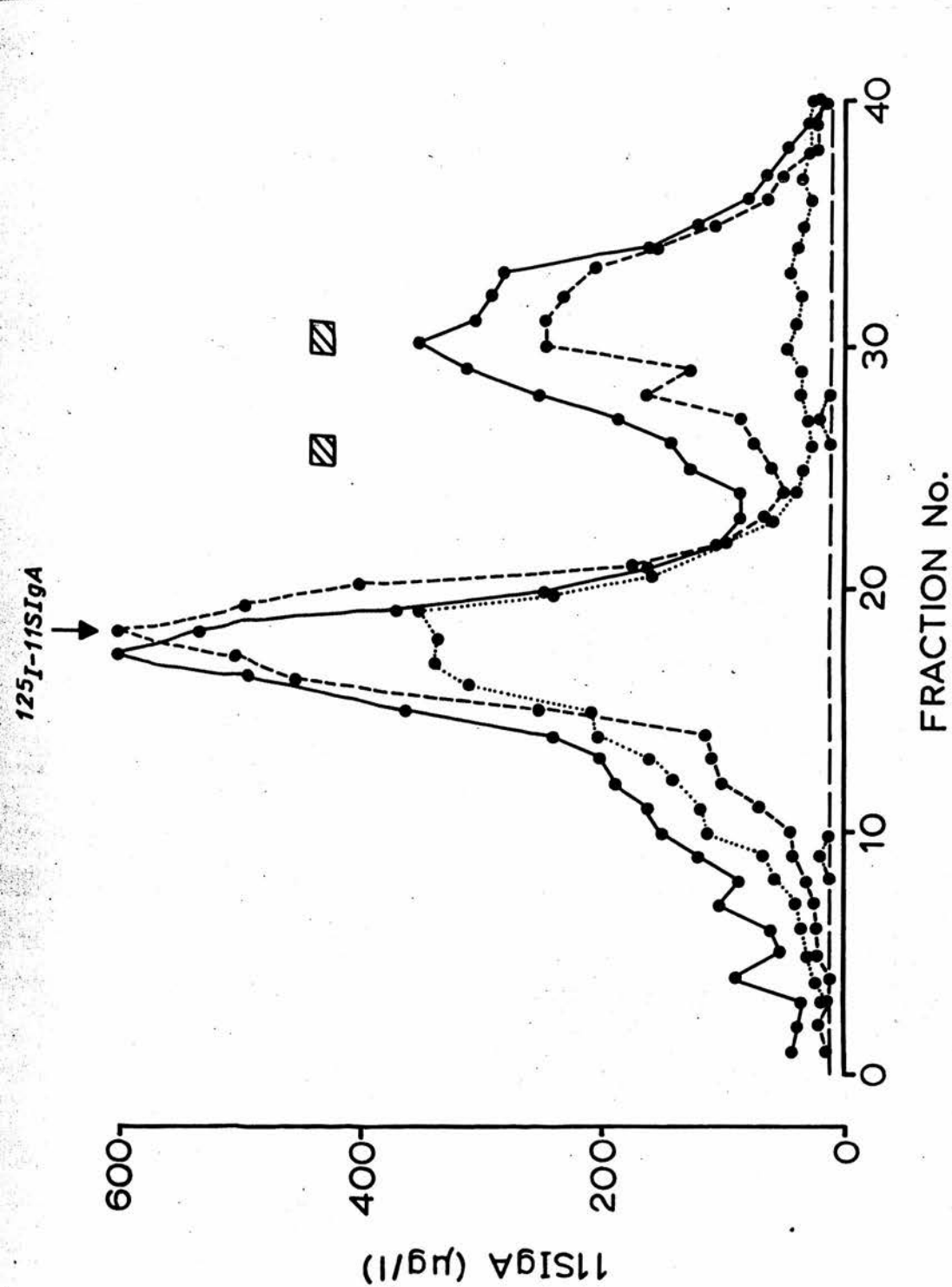


Fig. 6.5: Sedimentation coefficient determination for colostrum (—●—), milk (---○---), galactorrhoeic secretion (.....●.....) and serum (.....○.....). The same fractions as described in Fig. 6.4 were immunoassayed instead with an 11S IGA standard, and rabbit anti-11S IGA/FSC which does not have antibody activity against 7S IGA and therefore only detects 11S IGA and free secretory component.

milk and ^{125}I -11S IgA marker, but differed in position from the immunoreactivity peak for normal serum, which coincided with the position of the 7S marker. This suggested that the IgA present in galactorrhoeic secretions is 11S (Fig. 6.4).

Further immunoassay of the same fractions, but with the rabbit anti-11S IgA/FSC, demonstrated a single peak of immunoreactivity for the same galactorrhoeic secretion at the 11S position, confirming that the IgA present in galactorrhoeic secretions was a dimer combined with secretory component and not simply a 10S dimer. In the case of colostrum and milk, two peaks of immunoreactivity were seen, one coinciding with the ^{125}I -11S IgA marker, and the other coinciding with the non-radioactive 4.5S marker. This more slowly sedimenting peak of immunoreactivity had a sedimentation coefficient value similar to that of free secretory component (Tomasi and Bienenstock, 1968) but accurate quantitation was not possible due to the lack of a suitable free secretory component standard. No peaks of immunoreactivity were detected with the fractions from ultracentrifugal analysis of normal adult serum, indicating no detectable 11S IgA or free secretory component in this particular assay (Fig. 6.5) and the complete adsorption of the antiserum with 7S IgA.

Gel Filtration

Gel filtration of two different samples of galactorrhoeic secretions resulted in a single peak of immunoassayable IgA (Fig. 6.6). This IgA eluted at the same volume as the IgA peak in colostrum or milk (Fig. 4.8) but at a different volume from the IgA peak of normal adult serum (Fig. 4.9), confirming once again that the IgA present in galactorrhoeic secretions was 11S. The 11S IgA standard was therefore used in IgA assays of galactorrhoeic secretions.

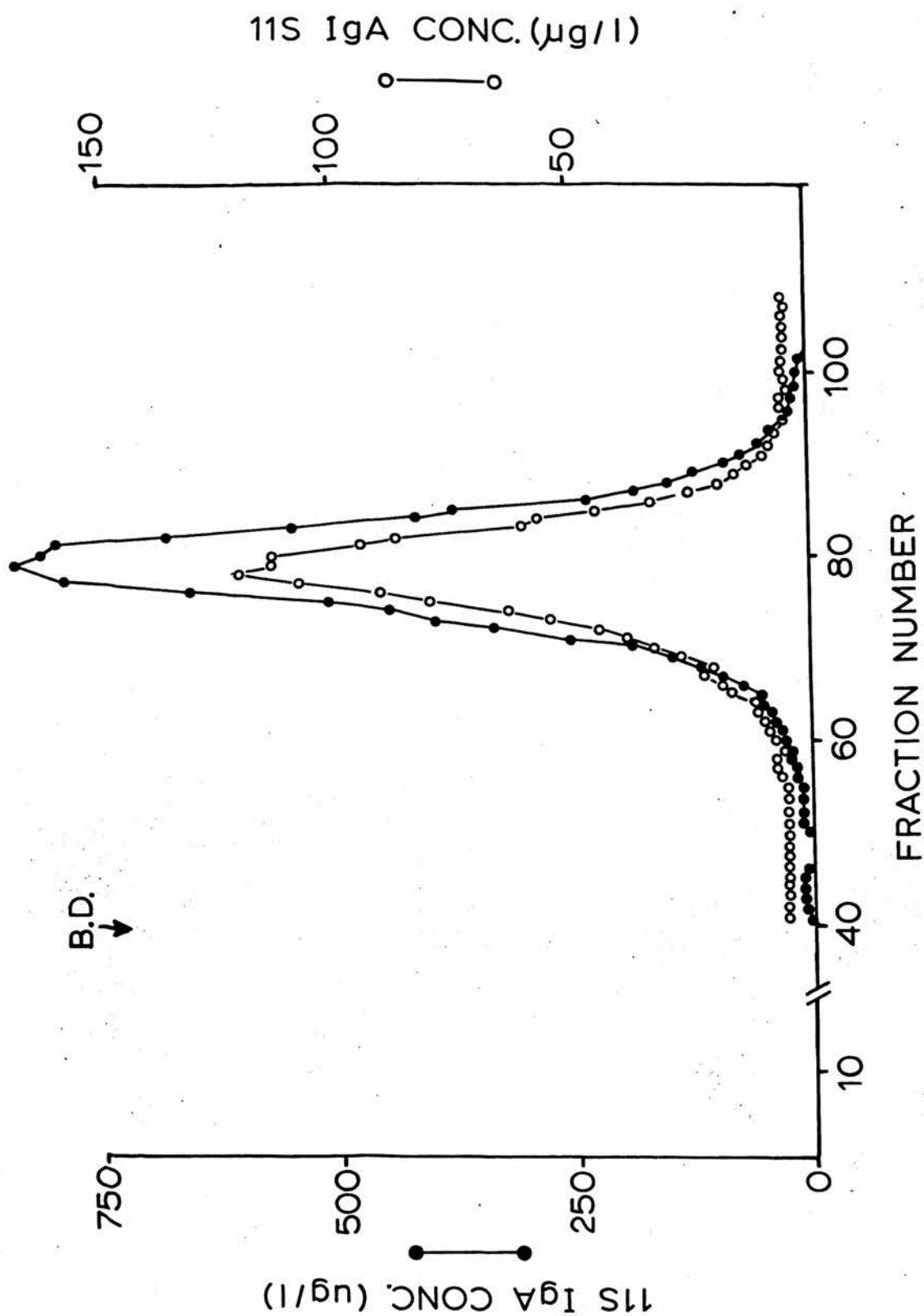


Fig. 6.6: Gel filtration on a Sepharose 4B column of galactorrhoeic secretion from patients no. 1 (●—●) and no. 2 (○—○). 3 ml fractions were collected and assayed by R.I.A. for IgA using an 11S IgA standard. The arrow indicates the elution volume of a Blue Dextran marker (B.D.; void volume), and the same experimental conditions were used as in Fig. 4.8 (colostrum and milk) and Fig. 4.9 (normal adult serum).

Concentrations of Milk Proteins in Galactorrhoeic secretions

The concentrations of the various milk proteins found in the four samples of galactorrhoeic secretions were compared with concentrations of milk proteins in colostrum and day 6 milk determined previously by McClelland *et al.* (1978) and are shown in Table 6-4. In all four patients studied, the milk protein concentrations observed were comparable with those found in colostrum. The ratio of IgA present in galactorrhoeic secretions to IgA present in serum was very much greater than the corresponding ratios for IgG and albumin (Table 6-4).

6.3.3 Discussion

Previous studies of the composition of the mammary secretions of patients with galactorrhoea have mainly concentrated on measuring total fat, carbohydrate and protein concentrations (Levine *et al.*, 1962; Brown *et al.*, 1965; Vorherr, 1975). In addition, some studies have indicated that casein (Brown *et al.*, 1965) and α -lactalbumin (Kleinberg *et al.*, 1977) are present; the concentration of lysozyme (Brown *et al.*, 1965) has also been measured in galactorrhoeic secretions. Unfortunately, in these earlier studies, the serum prolactin concentrations in the patients studied were not specified nor were highly specific methods of assay used.

In this study, mammary secretions from hyperprolactinaemic patients with galactorrhoea were compared with normal post-partum colostrum or milk, so as to study prolactin-induced secretory changes in the breast in isolation from changes due to human placental lactogen or placental steroids. In three patients, (nos. 1, 2 and 4) prolactin was acting alone, unlike in patient no. 3 where galactorrhoea had persisted post-partum, and the breast had undergone various steroid hormone, placental lactogen and prolactin induced changes.

Table 6-4: Milk protein concentrations in galactorrhoeic secretions, post-partum colostrum, milk and maternal serum

Patient	11S IgA g/l	Breast secretion IgA serum IgA	IgG g/l	Breast secretion IgG serum IgG	Lactoferrin g/l	Lysozyme g/l	Albumin g/l	Breast secretion albumin serum albumin
1	55.10	44.44	3.20	0.56	25.00	0.42	1.86	0.05
2	69.57	56.10	1.60	0.28	46.50	2.40	9.20	0.26
3	40.64	32.77	1.25	0.22	18.50	0.48	5.62	0.16
4	32.36	26.07	0.05	0.01	2.19	0.80	2.47	0.07
colostrum*	120.47 ⁺ 26.9	97.15	0.34 ⁺ 0.07	0.06	14.82 ⁺ 1.94	0.46 ⁺ 0.14	1.93 ⁺ 0.34	0.06
milk*	3.68 ⁺ 0.39	2.97	0.04 ⁺ 0.02	0.01	4.95 ⁺ 0.26	0.12 ⁺ 0.02	0.36 ⁺ 0.02	0.01
serum**	1.24		5.69		N.D.	0.008	34.86	

* Data from McClelland et al. (1978). Colostrum and milk samples were collected on days 1 and 6 post-partum respectively and the data is expressed as mean ± S.E.M. (n = 53 and 31).

** Unpublished observations by McClelland and Samson on venous blood collected from mothers post partum (n = 19). IgA was determined using a 7S IgA standard.

The predominant form of IgA present in galactorrhoeic secretions has a sedimentation coefficient and a molecular size similar to 11S IgA in normal colostrum and milk, as demonstrated by density gradient ultracentrifugation and gel filtration. In addition, by using anti-11S IgA/FSC, the IgA detected in galactorrhoeic secretions is a combination of IgA with secretory component rather than a dimer (10S) of serum IgA. It is not possible to explain the lack of complete parallelism in the inhibition of ^{125}I -11S IgA binding between galactorrhoeic secretions and the 11S IgA standard. It may be due to the presence of higher molecular weight polymers of IgA, similar to those described in early colostrum by Wadsworth (1978) using the more sensitive technique of immuno-gel filtration.

The IgA in galactorrhoeic secretions could be derived from either selective or passive transfer from serum, or from local synthesis. In external secretions, the majority of IgG and albumin molecules are derived from serum by passive transfer (Brandtzaeg *et al.*, 1970). Furthermore, only small quantities of 11S IgA are present in human serum (Waldman, Mach, *et al.*, 1970). As the ratio of IgA present in galactorrhoeic secretion to IgA present in serum is so much greater than the corresponding ratios for IgG and albumin, it is likely that the high concentrations of 11S IgA that were detected were due to local synthesis as in the normal post-partum lactating breast.

The reason for the apparent absence of free secretory component from galactorrhoeic secretions is not clear. However, as it is not feasible to quantify free secretory component at the present time (due to a lack of the purified protein standard) no further comment on this result is possible.

The lactoferrin and lysozyme present in external secretions originates from glandular acini (Masson *et al.*, 1966) and epithelium

(Kraus and Mestecky, 1971) respectively. The demonstration of high concentrations of lactoferrin and lysozyme, similar in magnitude to the concentrations observed in colostrum and milk (McClelland et al., 1978) indicates that the effect of hyperprolactinaemia is to cause either epithelial proliferation, increased synthesis by epithelial cells or both. This may be of importance in understanding the transit of IgA precursor plasma cells to the human breast, as Weiss-Carrington et al., (1978) suggest that the migration to, and maturation in the mammary gland of the plasma cells of the mouse may be due to some factor produced by epithelial cells following stimulation by the hormones of pregnancy. These workers found that prolactin was the most important of the hormones of pregnancy in causing an elevation of IgA plasma cells in the mammary gland and of intra-epithelial IgA. However, to obtain a maximal elevation of IgA plasma cells, progesterone and oestrogen were also required.

One of the patients studied (no. 4) was nulliparous and amenorrhoeic, and therefore it was likely that in this patient, ovarian steroids played a minor role, if at all, in 'attracting' IgA plasma cells to the breast. In the two patients who were parous (no. 1 and 2), involution of the breast had occurred before the onset of galactorrhoea, again suggesting that ovarian steroids have a minor role in IgA precursor cell 'attraction' since it is known that in the human, post-lactation involution of the breast is accompanied by a decrease in the number of plasma cells (Pumphrey, 1977). In addition, in patient no. 1, the long duration of the galactorrhoea suggests that there must have been further migration of IgA precursor plasma cells to the breast since it is known that in the mouse, the maximal life span of an intestinal IgA plasma cell is eight weeks

(Mattioli and Tomasi, 1973). In the remaining patient (no. 3) the galactorrhoea persisted post-partum, and it is therefore possible that in her case, the migration of IgA precursor lymphocytes might have been initially influenced by ovarian and placental steroids and placental lactogen.

In conclusion, the data shows that in the human, as in the mouse, prolactin, possibly acting via the mammary epithelium causes a rise in 11S IgA secretion by the breast. The actual factor that accounts for the migration and maturation of plasma cells in the breast remains unidentified (Lamm, 1976) but may be related to some property of the mammary epithelium (Wiman, Curman, Forsum, Klareskog, Malmnäs-Tjennlund, Rask, Trägårdh and Peterson, 1978).

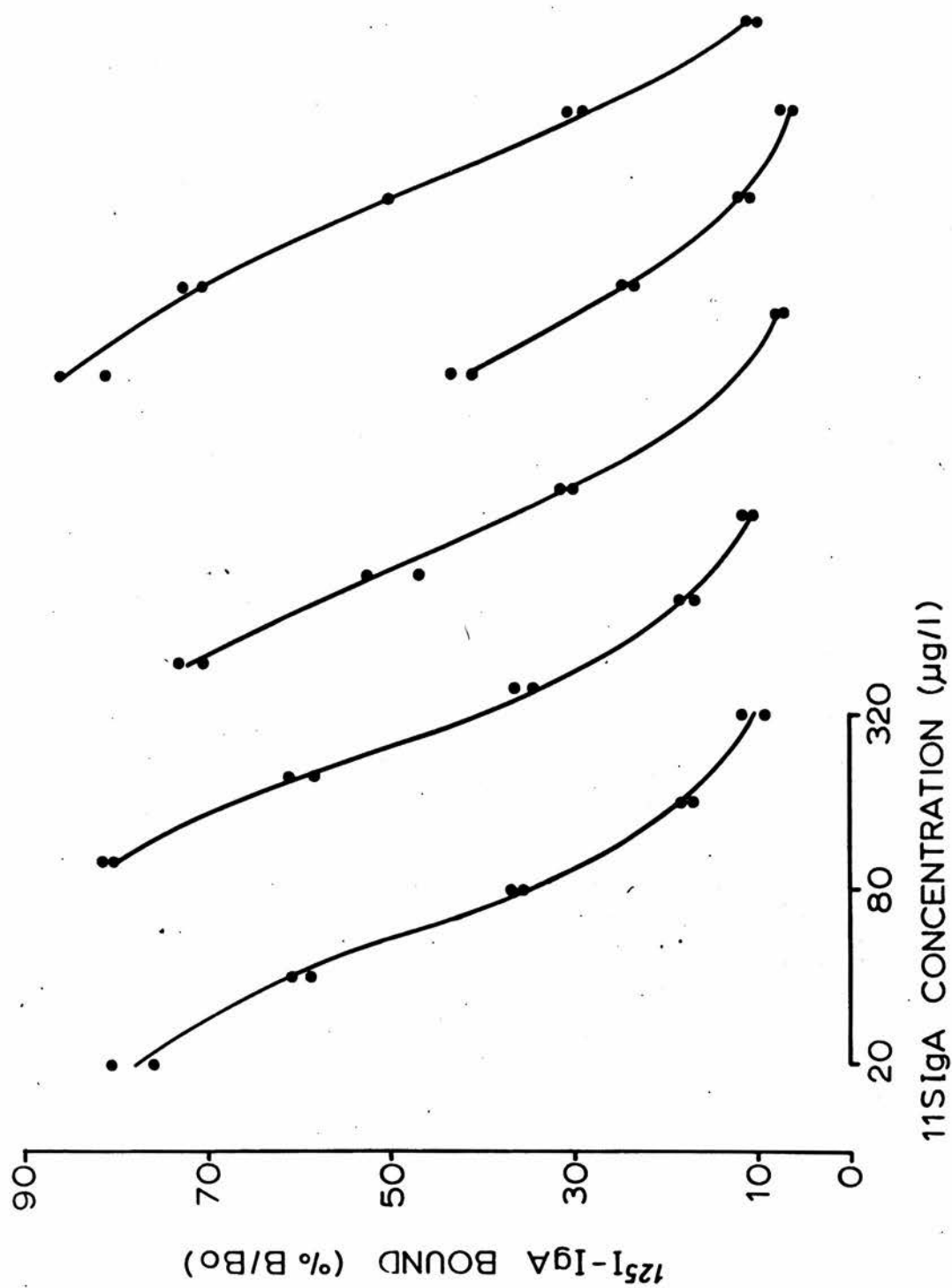


Fig. 6.7:
 ^{125}I - ^{11}S IgA binding inhibition curves for (from left to right) the ^{11}S IgA standard, and samples of normal breast secretions from each of four women.

6.4 STUDIES ON NORMAL BREAST SECRETIONS

6.4.1 Patients and Methods

Breast secretions: Breast secretions were collected by nipple aspiration by Dr. R.J. Crael, Mr. V. Humeniuk and members of the University Department of Clinical Surgery using a suction cup as described by Sartorius (1973). Details of subjects and clinical data (where known) are described in Table 6-5. The volume of secretions ranged from 15 to 215 μ l and were stored diluted in phosphate buffer at -20°C until assay. In three subjects (nos. 3, 5 and 10), secretions were obtained from different ducts within the same breast.

Quantitation of IgA and IgG

IgA was measured by RIA using a purified 11S IgA standard. IgG was also measured by RIA using a serum IgG standard that had been standardised against the W.H.O. standard (Rowe et al., 1972).

Quantitation of lactoferrin

Lactoferrin was measured by single radial immunodiffusion as described in the previous section (Section 6.3.1).

Gel filtration

Gel filtration was carried out in an identical manner to the method described in the previous section (Section 6.3.1). The fractions that were collected were immunoassayed using either rabbit anti-7S IgA or anti-11S IgA/FSC.

6.4.2 Results

Standard inhibition curve for IgA

The binding of ^{125}I -11S IgA was displaced in a parallel manner by the 11S IgA standard and breast secretions from each of four subjects, suggesting that immunoassayable 11S IgA was present (Fig. 6.7)

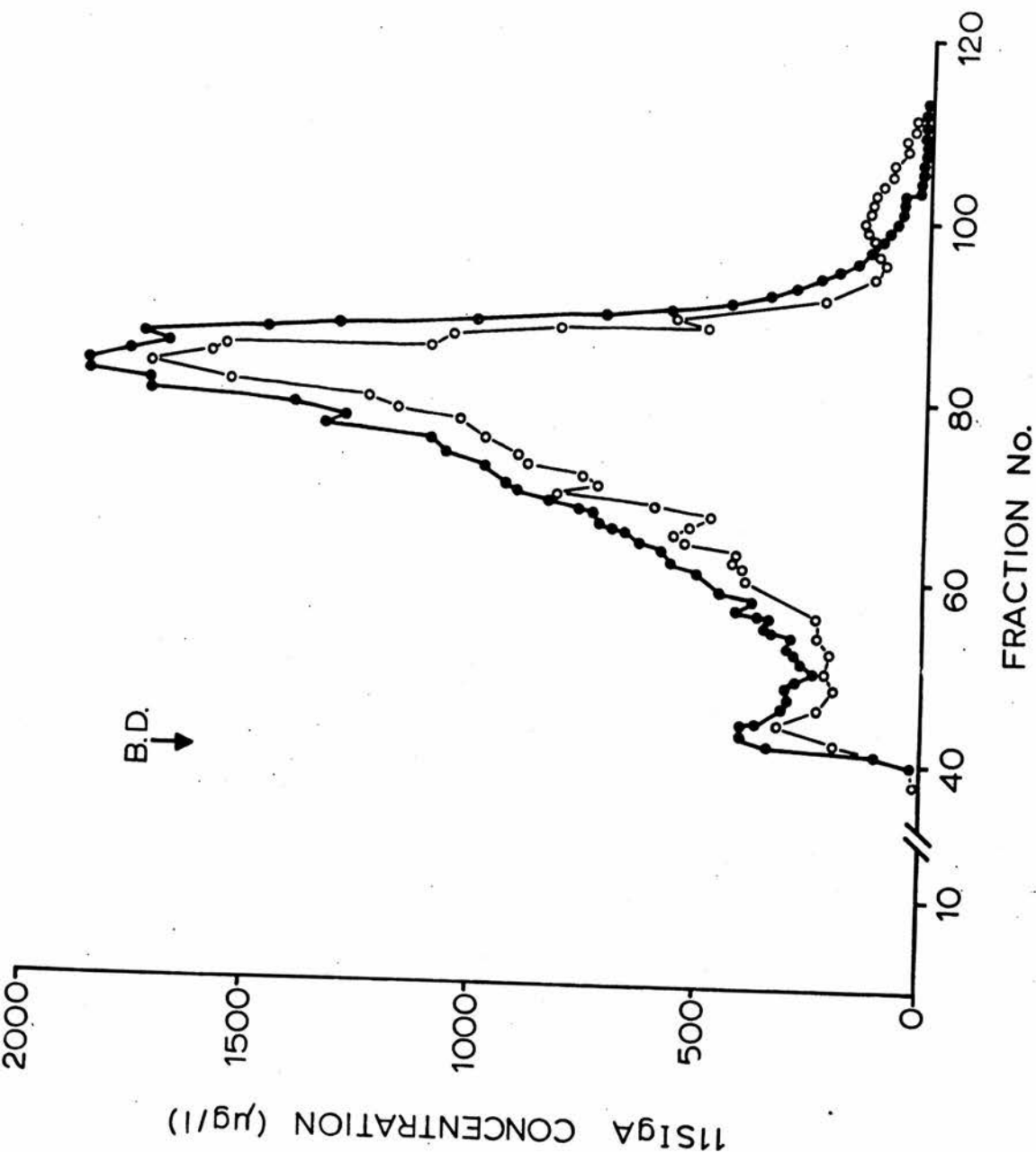


Fig. 6.8:

Gel filtration on a Sepharose 4B column of a normal breast secretion. 3 ml fractions were collected and assayed by RIA using an ^{11}S IgA standard, and either rabbit anti- ^{11}S IgA (●—●) which detects all forms of IgA, or anti- ^{11}S IgA/FSC (○—○) which detects only ^{11}S IgA and free secretory component. The arrow indicates the elution volume of a Blue Dextran marker (B.D.; void volume), and identical experimental conditions were used as in Fig. 4.8 (colostrum and milk) and Fig. 4.9 (normal adult serum).

Gel filtration

Gel filtration of the breast secretion from subject no. 3 resulted in a major peak of IgA immunoreactivity (Fig. 6.8) with an elution volume identical to the elution volume of the IgA peak in colostrum and milk (Fig. 4.8) but different to the elution volume of the IgA peak of normal adult serum (Fig. 4.9), confirming that IgA in normal breast secretions was predominantly 11S. Higher molecular weight forms of IgA were present also, as demonstrated by the immunoreactive material eluting after the void volume, but before the major peak (Fig. 6.8).

To confirm that immunoreactive material detected was immunologically identical to 11S IgA, the same fractions were also assayed using anti-11S IgA/FSC. An identical elution profile was found, indicating that all the immunoreactive material eluting before fraction 85 was probably 11S IgA in an aggregated form, and confirming again that the major peak of IgA immunoreactivity was due to 11S IgA and not 7S IgA (Fig. 6.8).

Concentrations of 11S IgA and IgG in Normal Breast Secretions

11S IgA and IgG were detected in all the breast secretions obtained from normal subjects: values ranged from 4.5 to 237.6 g/l and 1.4 to 67.1 g/l respectively. The mean (\pm S.E., $n = 18$) 11S IgA concentration of 117.2 ± 15.3 g/l was significantly higher than the mean IgG concentration of 7.3 ± 3.7 g/l ($p < 0.001$; paired t-test), but no significant correlation existed between 11S IgA and IgG values for each individual secretion (Table 6+5).

In an attempt to assess the validity of the immunoglobulin concentrations observed in breast secretions, the value for the right breast was compared with the value obtained for the left breast, where

Table 6-5: IgA and IgG in normal breast secretions

Subject	Age	Menstrual status	Parity	Secretion no.	IgA concentration (g/l)	IgG concentration (g/l)	Comments
1	50	Menopausal	Not known	15R	45.58	0.75	Ca of other breast
2	42	Pre-menopausal	Not known	19R	114.73	2.50	Normal
3	52	Post-menopausal	Parous	20L ₁	122.78	2.81	Normal
				20L ₂	116.98	2.89	
				20R	125.80	3.26	
4	25	Pre-menopausal	Parous	22L	10.28	2.11	Mastalgia
				22R	119.25	6.45	
5	49	Meno-pausal	Parous	23L ₁	6.00	1.28	Normal
				23L ₂	4.52	1.45	
				23R	10.82	1.91	
6	Not known	Post-menopausal	Not known	24L	160.50	67.08	Normal
7	24	Pre-menopausal	Not known	29L	188.25	5.59	Galactor-rhoea
				29R	108.50	2.52	
				33L	330.50	9.10	
				33R	243.25	3.08	
8	39	Pre-menopausal	Parous	30R	44.3	0.96	Mastalgia
9	41	Pre-menopausal	Parous	31L	300.0	2.51	Cystic disease, right breast
10	45	Pre-menopausal	Parous	32L	144.15	25.90	Normal
				32R	144.20	1.08	
				37L ₁	108.10	1.26	
				37L ₂	237.60	2.02	
				37R	108.80	1.05	
11	30	Pre-menopausal	Nulli-parous	34L	104.90	1.11	Normal
				34R	126.60	1.04	
				38	181.70	1.22	
12	35	Pre-menopausal	Not known	35	57.80	6.15	Normal
				39R	234.20	7.91	

Table 6-5 - continued

Subject	Age	Menstrual status	Parity	Secretion no	IgA concentration (g/l)	IgG concentration (g/l)	Comments
13	47	Pre-menopausal	Not known	227	70.08	1.94	Benign breast disease
14	49	Pre-menopausal	Not known	283L	8.62	1.14	Cystic disease
15	53	Menopausal	Parous	245R	178.40	2.20	Benign breast disease

The secretion number indicates the way in which the secretion was obtained e.g. for patient no 10, 32L and 32R were taken from left and right breasts respectively on the same occasion; on another occasion, 37L₁, 37L₂ and 37R were obtained, but the secretion within two separate ducts ending in the left nipple (L₁, L₂) were collected individually.

secretions were obtained from both breasts on the same occasion.

Where more than one secretion was obtained from a breast on a particular occasion (e.g. subject 10, secretions 37L₁, 37L₂), the mean value for the breast was used for the comparison. IgA values for the right breast ranged from 63 to 206% of the values seen for the left breast (mean \pm S.E. = $119 \pm 24\%$; n = 5) while corresponding IgG values showed a greater degree of variation (due to the IgG value for secretion 32L) ranging from 4 to ¹¹⁴139% (mean \pm S.E. = ⁶⁷83 \pm ¹⁸17%). In three subjects (nos.3, 5 and 10), the secretion obtained from each of two different ducts within the same breast was assayed separately. 11S IgA and IgG concentrations were very similar in two subjects (nos.3 and 5) but showed a two fold variation in the third (subject no.10) suggesting that a variation in the immunoglobulin concentration of secretions might occur between ducts in the same breast.

In view of the small number of subjects studied, and the incomplete clinical data, it was not possible to assess the relationship of age, menstrual status or parity to immunoglobulin concentrations of breast secretions. However, high concentrations of 11S IgA were found in the secretions obtained from postmenopausal or nulliparous subjects (nos.3 and 6; no 11). In addition, concentrations of 11S IgA were in general very similar to the range of concentrations seen in colostrum and milk (McClelland *et al.*, 1978; Table 6-4). The reason for the high concentrations of IgG detected in the secretions of subjects 6 and 10 is not clear.

Among the small number of secretions studied obtained from subjects with breast disease, all but two of the secretions (31L, 33L) were within the range of 11S IgA concentrations found for secretions from normal subjects. Similarly, all but two secretions

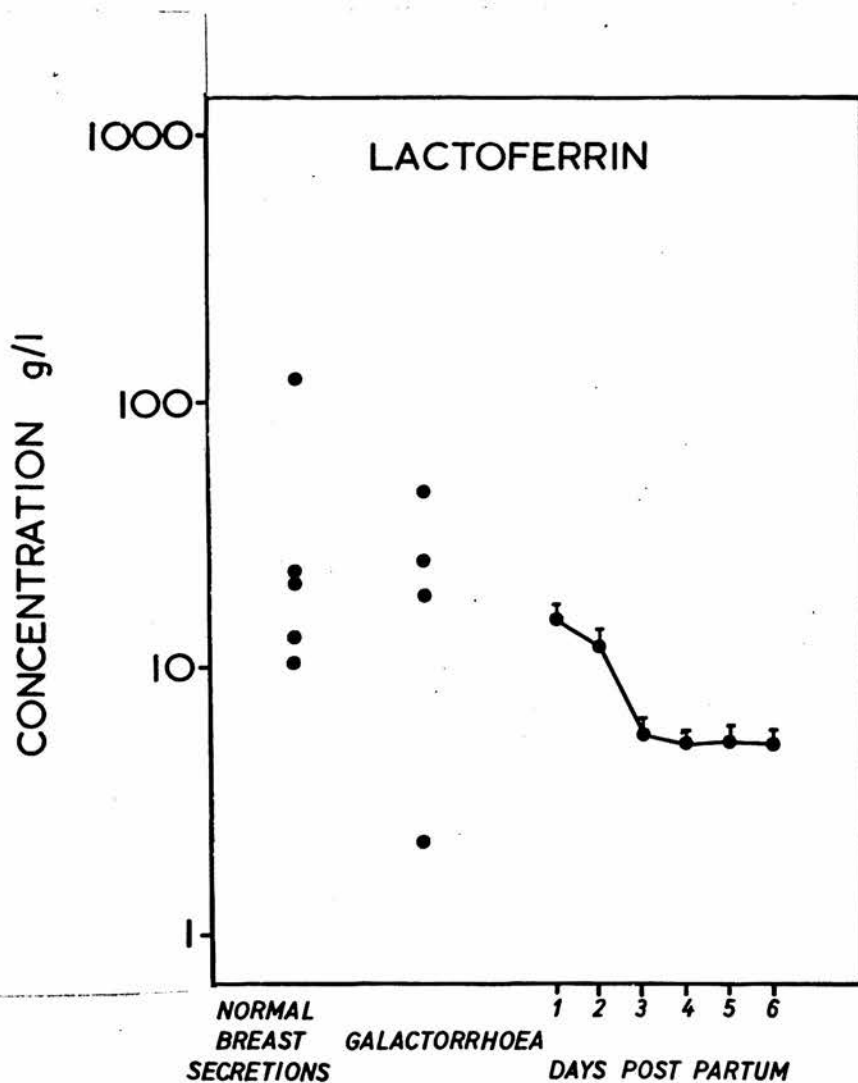


Fig. 6.9:

Scatter histogram comparing lactoferrin concentrations in (a) the normal breast secretions of 5 subjects (in whom lactoferrin was detectable), (b) the mammary secretions of four subjects with galactorrhoea (described in the preceding section), and (c) normal post partum colostrum and milk (data from McClelland *et al.*, 1978).

(15R, 30R) were within the range of IgG concentrations seen in the secretions of normal subjects. This suggests that no major differences are likely to be present between breast secretions obtained from normal subjects and those obtained from patients with breast disease.

Concentrations of lactoferrin in Normal Breast Secretions

Concentrations of lactoferrin similar to the concentrations found in colostrum and milk were detected in 5 out of 10 breast secretions obtained from three normal subjects (nos. 10, 11 and 12). The failure to detect lactoferrin in the remaining secretions was probably due to the dilution (1/200 to 1/500) of the secretion before assay (Fig. 6.9).

6.4.3 Discussion

Small amounts of secretion can be aspirated from at least 75% of non-lactating breasts (Petrakis et al., 1975) and previous studies on breast volume (Milligan et al., 1975), IgA synthesis (Drife et al., 1976) and DNA synthesis (Masters et al., 1977) suggest that the non-lactating breast responds to fluctuating levels of ovarian hormones. IgA concentrations were determined in secretions obtained from normal non-lactating breasts ('normal breast secretions') to gain insight into the influence of hormones associated with the menstrual cycle on the secretory immune system of the breast. In addition, lactoferrin concentrations were determined as an indicator of epithelial synthetic activity (Masson et al., 1966) and IgG concentrations as an indicator of the passive diffusion of serum proteins into external secretions (Brandtzaeg et al., 1970).

The results of the ^{125}I -11S IgA binding inhibition curves and gel filtration experiment indicate that the IgA in normal breast secretions is 11S. This was confirmed using anti-11S IgA/FSC which

indicated that it was the complete 11S IgA molecule that was detected, and not the 10S form. Interestingly, high molecular weight forms of 11S IgA ($> 400,000$ M.W.) were also detected by gel filtration, possibly representing aggregation of 11S IgA or the synthesis of high molecular weight IgA, as occurs in early colostrum (Wadsworth, 1978). Very little IgA immunoreactive material eluted at the same volume as 7S IgA.

Absolute concentrations of IgA and IgG in normal breast secretions are very similar to the values found in colostrum and milk (McClelland et al., 1978; Table 6-4). As in the two previous studies described in this chapter, the ratio of IgA in normal breast secretions to IgA in serum was much higher than the ratio of IgG in normal breast secretions to IgG in serum, indicating that the 11S IgA found in normal breast secretions was due to selective transport or local synthesis. As there is little 11S IgA in adult serum (Waldman et al., 1970), the 11S IgA detected must be a product of local synthesis. This confirms the earlier report of Drife et al. (1976) who detected IgA synthesis in 81% of cultures of normal human breast tissue.

A wide range of 11S IgA and IgG concentrations in normal breast secretions were observed and the mean value of 11S IgA and IgG concentrations was seventeen and three times higher respectively than the IgA and IgG concentrations reported in a similar study by Petrakis, Doherty, Lee, Mason, Pawson, Hunt and Schweitzer (1977). These differences may have been due to the different analytical methods used, as Petrakis et al., used rocket immunoelectrophoresis instead of RIA. Furthermore, Petrakis et al. (1977) did not report the use of an 11S IgA standard, an omission that might give rise to a three to

six-fold difference in IgA values (Schuurman, 1977; Greenberg, Rossen, Six, Baxter and Couch, 1978), and their assays were performed on undiluted samples, with possible loss of immunoglobulin by non-specific adsorption to the storage vessel.

In this study, IgA and IgG was detected in all samples of breast secretion (whether from normal or diseased breasts). In contrast, Petrakis et al. (1977) reported the absence of IgA and IgG in a number of normal breast secretions and secretions from cancerous breasts. This difference may be attributed to the use of a less sensitive method of detection of immunoglobulins (rocket immunoelectrophoresis) and it is clear that if a deficiency exists, it is a relative rather than absolute one.

Although high concentrations of 11S IgA were detected in the normal breast secretions, because the volume of the secretions was very small, the total 11S IgA synthesis by the non-lactating breast is very much less than that of the lactating breast. Only a fraction of the total secretion lying within the mammary ducts of the non-lactating breast is obtained by aspiration with the suction cup, and the values for IgA, IgG and lactoferrin observed should therefore be considered as being representative of the ductal secretion nearest the nipple, rather than of the entire breast.

For the secretions obtained from normal subjects on a particular occasion (whether from left or right breast), IgA and IgG concentrations within each patient vary up to two fold, with the exception of secretion 32L. This suggests that measurements of immunoglobulin concentrations in breast secretions may be a valid procedure despite the problem of obtaining uniform volumes of secretion.

It is thought that the basal activity of the epithelial cells in the normal human breast during the menstrual cycle may be due to prolactin, insulin and possibly oestrogen and progesterone (McNeilly, 1977). The high concentrations of lactoferrin observed are indicative of this basal activity, and in the mouse, it has been proposed that some unknown factor originating from epithelial cells may be responsible for the 'attraction' of IgA precursor lymphocytes into the mammary gland (Weisz-Carrington et al., 1978). The finding of high 11S IgA concentrations in the breast secretions of nulliparous (subject no. 11) or parous premenopausal subjects suggests that one or more of the hormones of the normal menstrual cycle may be influencing the secretory immune system of the normal breast. In two postmenopausal subjects (no. 3 and 6), high 11S IgA concentrations in the breast secretions were also observed, suggesting that the hormone influencing the influx of IgA precursor lymphocytes may still be secreted after the cessation of menstrual periods. As serum prolactin concentrations in postmenopausal women are only slightly less than those in eugonadal women (Reuter, Kennes, Gavaert and Franchimont, 1976), prolactin might be the hormone involved.

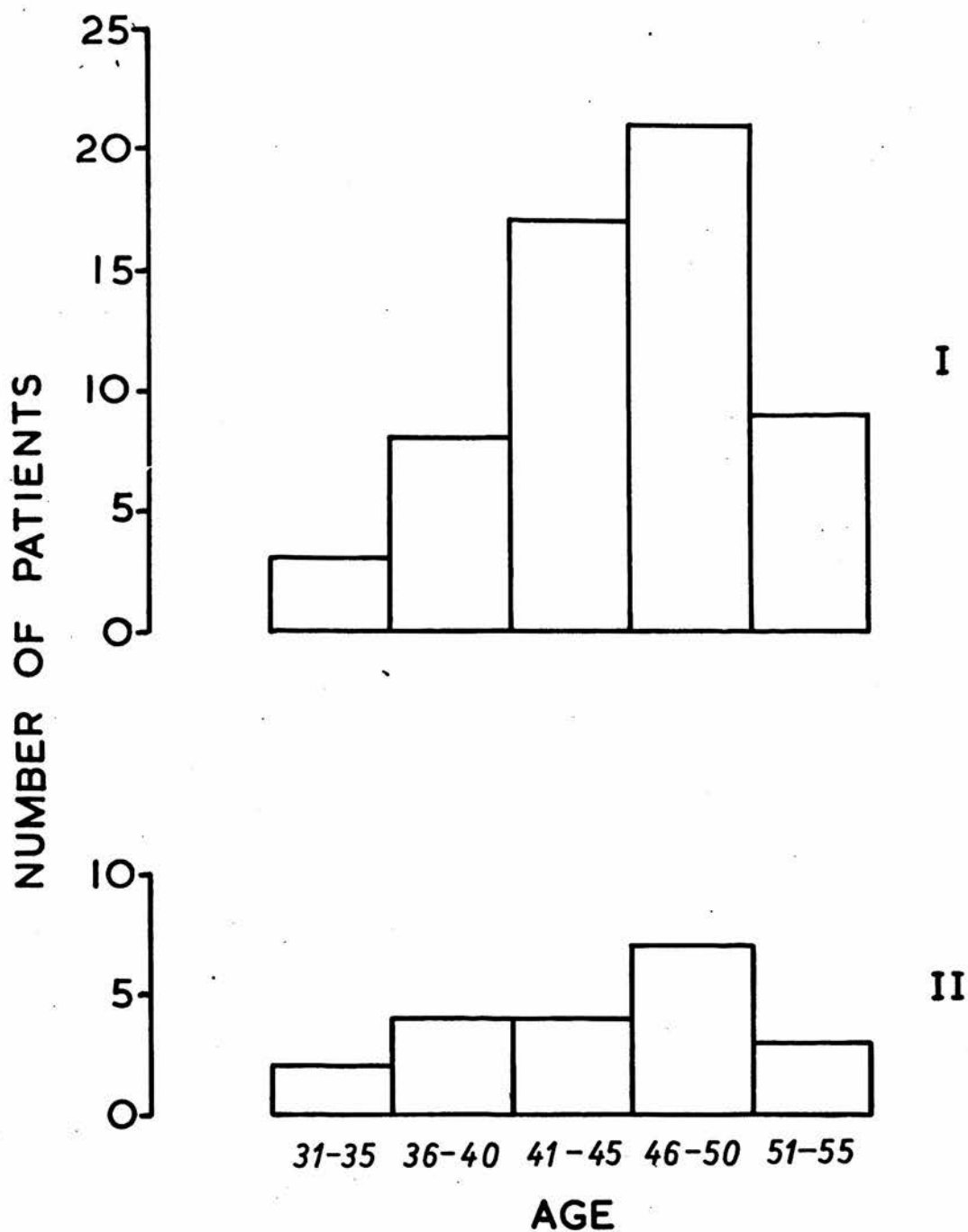


Fig. 6.10:

Age Distribution of patients studied with gross cystic disease of the breast. The cyst fluids of Group I patients were assayed within 2 years of collection while cyst fluids of Group II patients were assayed within 3 months of collection.

6.5 STUDIES ON CYST FLUIDS

6.5.1 Materials and Methods

Cyst fluids: Cyst fluids were obtained by needle aspiration from female patients presenting with cystic disease by Drs. M.M. Roberts, R.J. Craiel, Mr. Vlad Hemeniuk and members of the University Department of Clinical Surgery. Two groups of patients were studied: an initial series of 71 cyst fluids from 59 patients aged 32 to 55 years whose cyst fluids were stored for up to two years at -20°C prior to assay (Group I), and a second series of 26 cyst fluids from 21 patients aged 31 to 54 years whose cysts were stored for a maximum of three months at -20°C prior to assay. Group II cyst fluids were collected because it was thought that Group I cyst fluids might have been adversely affected by the long storage period.

The age distribution of patients in the two groups was very similar, with the maximum number of cyst fluids collected from patients aged 46 - 50 years, and the smallest number of cyst fluids collected from patients aged 31 - 35 (Fig. 6.10). Reliable clinical data was available for Group II patients, but unfortunately clinical data for Group I patients was incomplete.

Quantitation of IgA

IgA was measured by RIA using an 11S IgA standard.

Quantitation of IgG, lactoferrin, lysozyme and albumin

Single radial immunodiffusion was used to quantify IgG, lactoferrin, lysozyme and albumin using reagents and standards described previously (McClelland *et al.*, 1978).

Quantitation of Dehydroepiandrosterone sulphate (DHAS)

A double antibody RIA was performed by Dr. W.R. Miller using the method of Buster and Abrahams (1972).

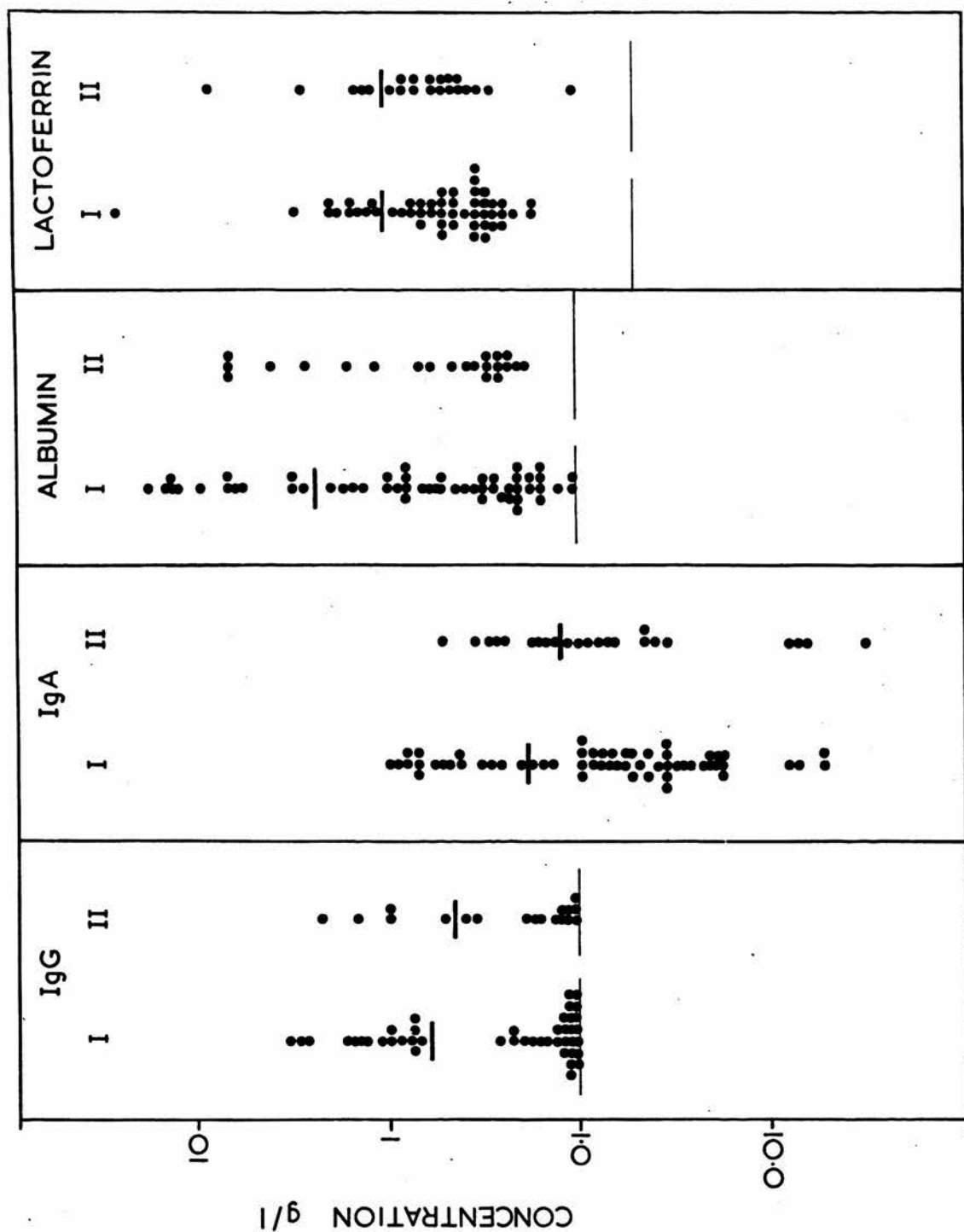


Fig. 6.11: Scatter histogram illustrating IgG, IgA (using an 11S IgA standard), albumin and lactoferrin concentrations in the cyst fluids of Group I and II patients. The thick bar indicates the arithmetic mean, and the thin horizontal bar indicates the limit of detection for the particular assay.

Gel filtration

Gel filtration was carried out on a single sample of cyst fluid in an identical manner to the gel filtration studies described in the previous section (Section 6.3.1).

Sedimentation Coefficient Determination

This was performed in a manner identical to the procedure described for the ultracentrifugal analysis of breast secretions from a patient with hyperprolactinaemic galactorrhoea (Section 6.3.1). A 7S IgA standard was used for the quantitation of IgA in ultracentrifugation fractions when the IgA detected was predominantly 7S, and the 11S IgA standard when the IgA detected was predominantly of 11S. In a few cyst fluids, fractions from ultracentrifugal analysis were also assayed using anti-11S IgA/FSC .

6.5.2 Results

IgA, IgG, lactoferrin, lysozyme and albumin concentration in cyst fluids

A wide range of concentrations of IgA (using an 11S IgA standard), IgG, lactoferrin and albumin were observed in cyst fluids (Fig.6.11). Lysozyme was only detected in 4 cyst fluids from among 95 examined, and concentrations ranged from 0.13 to 0.54 g/l. When the mean value for the concentrations of IgA, IgG, lactoferrin and albumin in Group I cyst fluids were compared with the corresponding mean values in Group II cyst fluids, no significant difference was found (Student's t-test), suggesting that the longer storage period for Group I cyst fluids had not adversely affected milk protein concentrations. The data for Group I and II cyst fluids was therefore pooled: mean (\pm S.E.) values for IgA, IgG, lactoferrin and albumin were 0.18 ± 0.03 g/l, 0.54 ± 0.09 g/l, 1.02 ± 0.29 and 2.29 ± 0.48 g/l

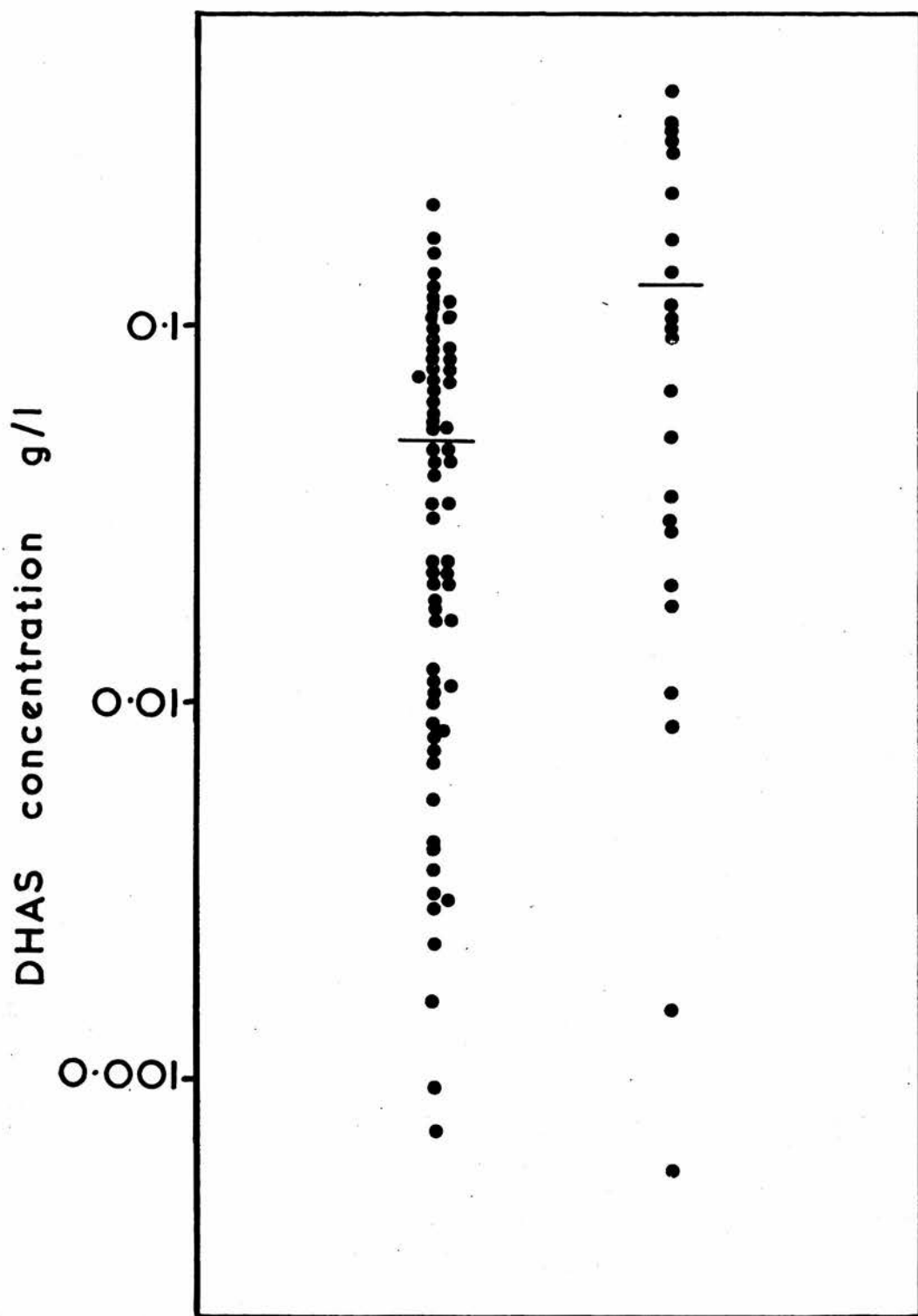


Fig. 6.12:

Scatter histogram illustrating DHAS concentrations in the cyst fluids of Group I (left histogram) and Group II patients (right histogram). The horizontal bar indicates the arithmetic mean.

respectively. The detection rate of these proteins was 100%, 68.4%, 88.4% and 94.7% respectively.

Three different cyst fluids were also examined from an additional patient with galactocoeles following a recent pregnancy. Mean (\pm S.E.) IgA, IgG, lactoferrin, lysozyme, albumin and DHAS concentrations were 2.99 ± 1.73 , 0.010 ± 0.003 , 2.02 ± 0.57 , 0.22 ± 0.17 , 0.60 ± 0.01 and $0.000,181 \pm 0.000,069$ g/l

DHAS concentrations in cyst fluids

A wide range of concentrations of DHAS-like material was detected in all cyst fluids (Fig.6.12) In contrast to the data for the milk protein concentrations above, the mean (\pm S.E.) values for DHAS concentrations in Group I and II cyst fluids was 0.051 ± 0.006 and 0.128 ± 0.131 g/l respectively, a difference that was highly significant ($p < 0.001$, Student's t-test). This suggested that the longer period of storage of Group I cyst fluids might have adversely affected DHAS concentrations.

Effect of Reaspiration of Cyst on concentrations of milk proteins and DHAS

Four cysts were aspirated at 21 day intervals in Group II patients to study the effect of successive aspirations on IgA, IgG, albumin and DHAS concentrations. It was found that in three of the patients, in particular patient no 4, marked changes in either IgG or albumin concentrations occurred. The data for the cyst fluids of three of the patients (nos 1, 2 and 4) on the second aspiration indicated that the aspiration procedure itself did not result in an increased albumin or IgG concentration at the second aspiration (Table 6-6).

Table 6-6: Effect of Reaspiration of Cyst after 21 days on cyst fluid

Patient	Cyst no.	Vol. (ml)	IgA concentration (mg/l)	IgG concentration (mg/l)	Albumin concentration (mg/l)	DHAS concentration (mg/l)
1	5	8.0	266	390	1100	93.00
	10	8.0	44	120	360	50.00
2	12	N.K.	42	130	550	269.00
	13	N.K.	32	< 100	300	348.00
3	18	N.K.	64	< 100	200	102.50
	19	N.K.	88	480	2500	115.00
4	22	5.0	275	1400	6400	1.51
	25	3.0	7	< 100	240	140.00

The second cyst fluid for each subject was aspirated 21 days after the initial aspiration of the cyst.

N.K. = not known.

Relationship of milk protein and DHAS concentrations to parity,
menstrual status and cyst volume

The parity of the patients studied in Group I and II (where known) ranged from parity 0 to parity 6. However, no obvious relationship was seen between milk proteins and DHAS concentrations, and parity. Unfortunately, no conclusion could be drawn about the relationship of menstrual status to cyst fluid concentrations of any of the substances studied because the majority of patients studied (approximately 90%) were premenopausal and there were insufficient numbers of menopausal and post-menopausal patients.

In the Group II patients, a record was made of the volume of the cyst fluids aspirated, but no obvious relationship was observed between the volume of the cyst fluid aspirated and the age, parity, menstrual status of the patient, or milk protein and DHAS cyst fluid concentrations.

Variation in IgA concentration in cysts from the same patient

Two cysts from each of two patients in Group II were aspirated on the same occasion. IgA concentrations in each pair of cyst fluids were 105 and 269 mg/l, and 75 and 169 mg/l. Unfortunately, it was not possible to measure other milk proteins or DHAS because of the small volume of cyst fluid available.

Gel filtration

A sample of cyst fluid with high IgA concentrations (no 26, Table 6-7) was analysed to ensure that the 11S IgA standard had been the correct one to use. It was found that the peak of IgA immunoreactivity in this particular cyst fluid coincided with the peak of immunoreactivity in a sample of normal adult serum analysed under the same experimental conditions. This suggested that cyst fluid no 26

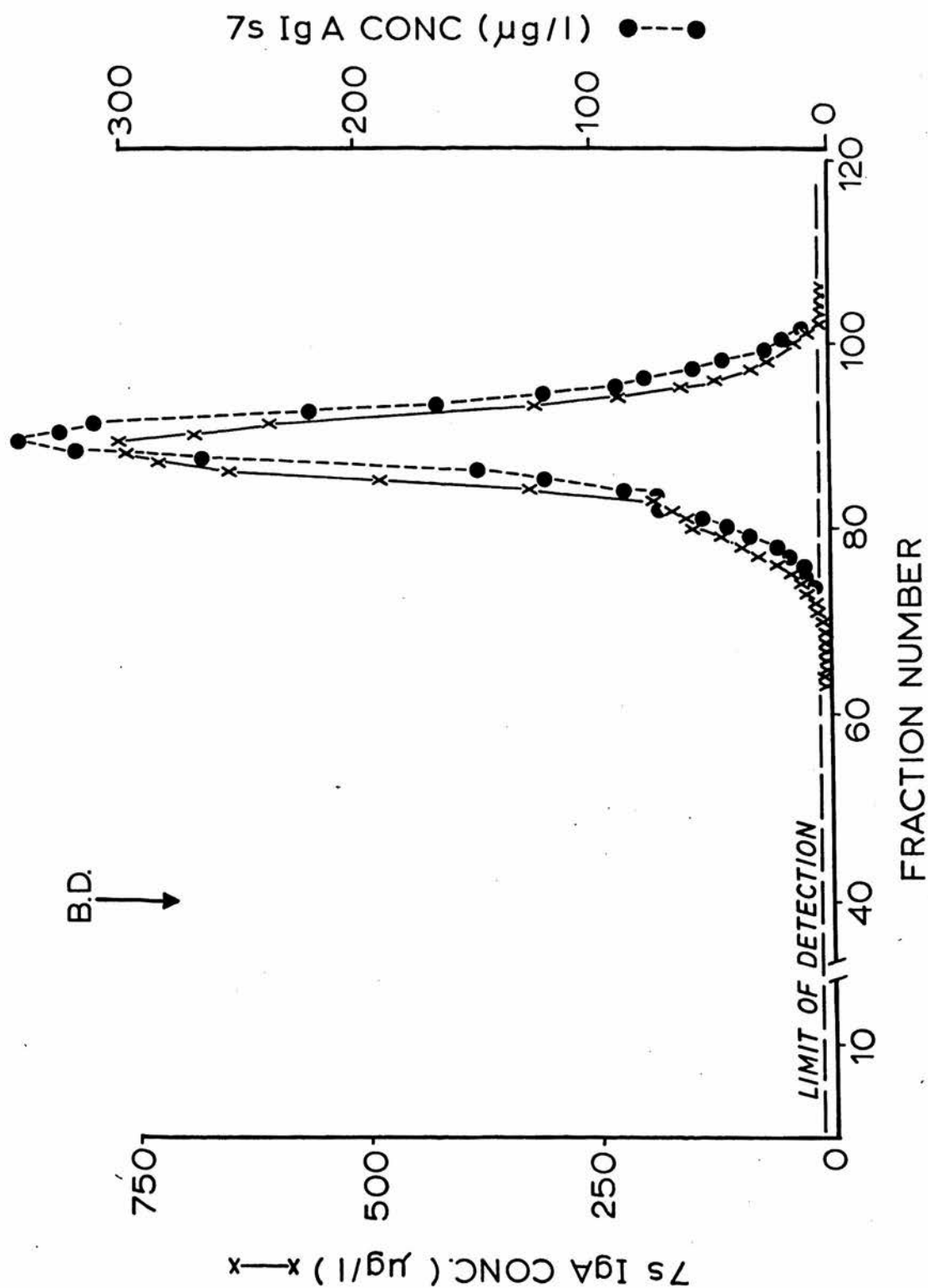


Fig. 6.13: Gel filtration on a Sepharose 4B column of a cyst fluid (●---●) from a patient with gross cystic disease of the breast demonstrating the presence of 7S IgA. 3 ml fractions were collected, and assayed by RIA using an ¹²⁵I IgA standard. The arrow indicates the elution volume of a Blue Dextran marker (B.D.; void volume). The elution profile of normal adult serum (x—x), as shown in Fig. 4.9, is illustrated for comparison. Experimental conditions were identical to those described for Fig. 4.8 (colostrum and milk).

contained mainly 7S IgA, and that the appropriate IgA standard to use for/cyst fluid was 7S rather than 11S (Fig. 6.13).

Sedimentation Coefficient Determination of IgA in Cyst Fluids

Sedimentation coefficient determinations were performed on the IgA present in the 19 cyst fluids with the highest IgA levels (from both Group I and Group II patients) where there was sufficient material for analysis. This was to confirm the finding of 7S IgA in a single cyst fluid (by gel filtration) and to ensure that the correct standard was being used for the measurement of IgA by RIA in the cyst fluids.

It was found that the peak of IgA immunoreactivity in nine of the cyst fluids studied was in the same position in the sucrose gradient as the 7S marker, while in the remaining ten, it was in the same position as the 11S marker. The IgA in fluid from the galactocoele was, as expected, in the 11S position (Fig. 6.14). Mixtures of 7S and 11S IgA immunoreactivity were seen in some cyst fluids (Fig. 6.15).

To confirm that the immunoreactive IgA in the same position as the 11S marker was truly 11S IgA, and not 10S IgA, some fractions were reanalysed, but using anti-11S IgA/FSC. It was found that a cyst fluid that had previously been found to have a peak of IgA immunoreactivity in the 11S position again showed a peak of immunoreactivity in the 11S position using anti-11S IgA/FSC. The fluid from the galactocoele also showed a peak of IgA immunoreactivity in the 11S position using anti-11S IgA/FSC, confirming that in these two fluids it was the complete 11S IgA molecule that was being detected. Interestingly, in these two fluids, small amounts of immunoreactive material were detected near fraction 35, a position on the sucrose gradient with a sedimentation coefficient value corresponding to free secretory component. However, precise quantitation of the free secretory component in these two cyst fluids

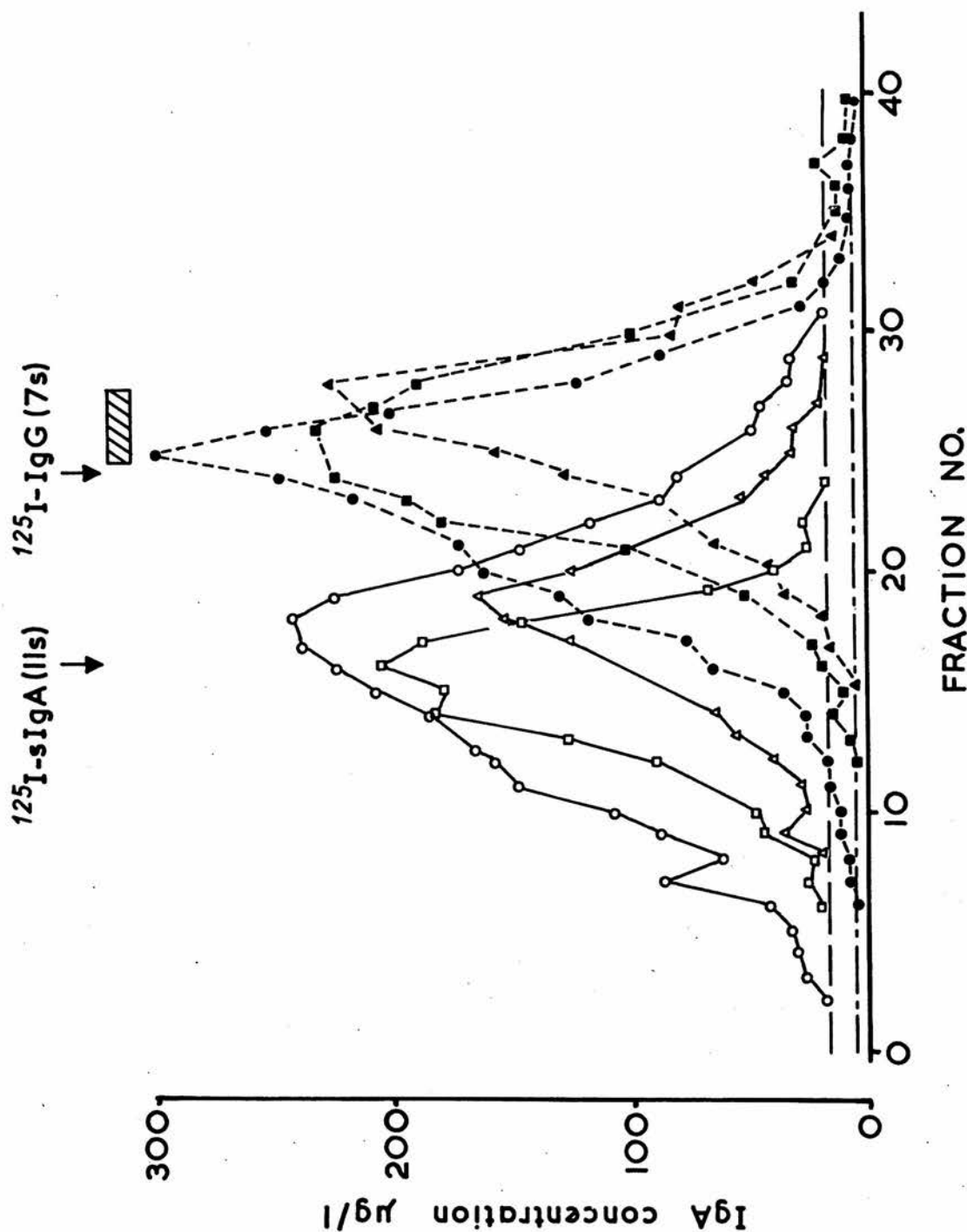


Fig. 6.14:

Sucrose density gradient ultracentrifugation to determine the sedimentation coefficient of IgA in five samples of cyst fluid (8(1), Δ ; 13(2), \circ ; 26, \blacksquare ; 31, \blacktriangle ; 57, \bullet ; see Table 6-7) and a sample of fluid from a galactocoele (\square). A 25-45% w/v gradient was used, and 0.1 ml fractions collected from the bottom of the tube and immunoassayed using an 11S IgA standard (cyst fluid 8(1), 13(2) and galactocoele fluid) or a 7S IgA standard (cyst fluid 26, 31 and 57) and anti-7S IgA. The two arrows indicate the position of radioactive 11S and 7S markers, and the hatched area a non-radioactive 7S marker.

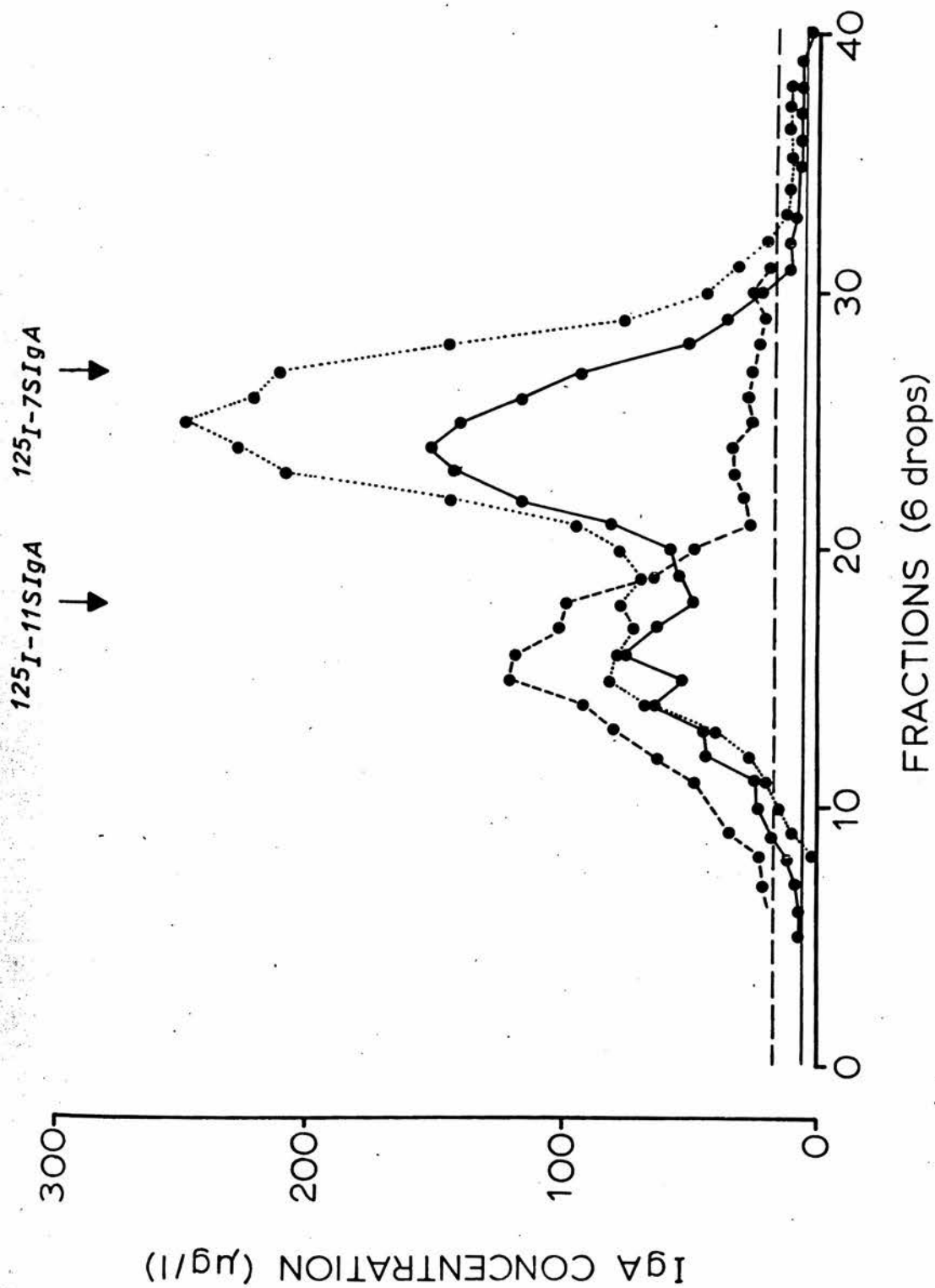


Fig. 6.15:

Sucrose density ultra centrifugation to determine the sedimentation coefficient of IgA in three samples of cyst fluid (5(2), 48, 26*); see Table 6-7) demonstrating that mixtures of 7S and 11S IgA can occur in the same cyst fluid. Anti-7S IgA was used, with a 11S IgA standard (cyst fluid 5(2)) or a 7S IgA standard (cyst fluid 48, 26*). The two arrows indicate the position of radioactive 11S and 7S markers. Experimental conditions were identical to those described for Fig. 6.14.

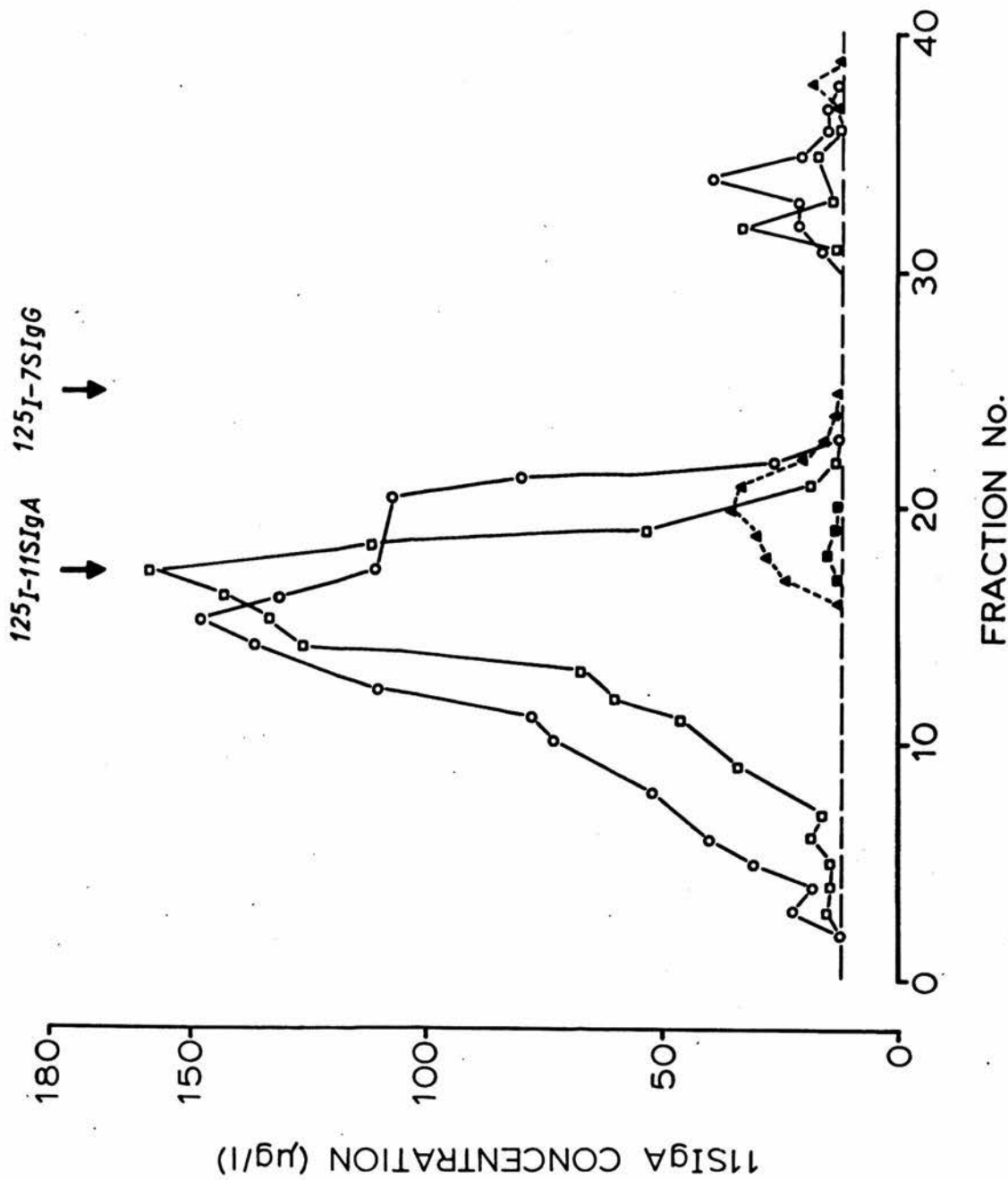


Fig. 6.16: Sedimentation profile of three cyst fluids (13(2), 26, 31) and fluid from galactocoele when the fractions described in Fig. 6.14 were immunoassayed using anti-11S IgA/FSC (which only detects IgA and free secretory component) and an 11S IgA standard. It was found that free secretory component was present, and that two cyst fluids (nos. 26 and 31) also contained traces of immunoreactive material similar to 11S IgA.

was not possible due to the lack of the purified protein standard. Although no peaks of IgA immunoreactivity were detected in the 7S position using this antiserum, it was observed that two of the cyst fluids with the peak of IgA immunoreactivity in the 7S position, also contained some immunoreactive material that suggested the presence of 11S IgA (Fig. 6.16).

The 19 cyst fluids that were analysed by sucrose density gradient ultracentrifugation were therefore divided into cyst fluids where the IgA was wholly or predominantly 7S or 11S. When the two categories of cyst fluids were compared, it was found that mean IgG and albumin concentrations were significantly greater in the cyst fluids where IgA was of the 7S form than in the cyst fluids where IgA was of the 11S form. In contrast, mean DHAS concentrations were significantly lower in the cyst fluids where IgA was of the 7S form ($p < 0.001$ in all cases, t -test; Table 6-7). When the clinical data for the two types of cyst fluids were compared, all the cyst fluids with IgA in the 11S form were noted to have been aspirated from premenopausal patients (Table 6-8).

6.5.3 Discussion

In this section, fluid obtained from the cysts of patients with gross cystic disease of the breast were studied because of the possibility that the fluid within the cyst might reflect the synthetic activity of the surrounding breast tissue, and therefore allow the study of the influence of various clinical factors on the secretory immune system of the breast. In addition, it was thought that a study of milk proteins and DHAS in cyst fluid might lead to an understanding of the mechanism of cyst formation.

Table 6-7: Classification of cyst fluids by the sedimentation coefficient of the major IgA peak

IgA Peak in 7S Position

Cyst fluid no.	IgA concentration (mg/l)	IgG concentration (mg/l)	Albumin concentration (mg/l)	DHAS concentration (mg/l)	Lactoferrin concentration (mg/l)
4	796	740	6400	0.95	440
26	669	2900	6400	11.00	25000
31	639	1600	12200	3.50	640
37	664	1500	13950	10.30	400
42	867	720	1820	3.00	230
48	286	1350	8900	2.30	690
57	676	3100	17050	18.00	1010
61	939	2750	13050	4.22	300
26*	334	2150	6400	0.57	1320
mean	652	1868	9574	5.98	3330
S.E.	73	300	1602	1.95	271

IgA Peak in 11S position

5 (2)	390	N.D.	200	48.00	1080
8 (1)	780	N.D.	200	80.00	1240
8 (2)	475	N.D.	200	53.50	1240
10 (1)	499	N.D.	70	57.50	1840
10 (2)	407	100	270	56.00	1730
13 (2)	776	100	120	23.50	720
18	432	N.D.	50	120.00	350
38 (1)	308	760	1170	3.15	580
44	422	110	470	42.50	1400
23*	493	N.D.	240	325.00	1200
mean	498	167	299	80.91	1138
S.E.	50	66	103	28.85	151
p	N.S.	< 0.001	< 0.001	< 0.001	N.S.

*Cyst fluids were all obtained from Group I patients except for two fluids marked with an asterisk, which were obtained from Group II patients. The figure in brackets indicates cyst fluid obtained from a different cyst, or on a different occasion, but from the same patient.

N.D. = Below the limit of detection (<100 mg/l). For statistical purposes, this was calculated as 100 mg/l.

N.S. = Not significant.

Table 6-8: Clinical data of patients with cyst fluids classified by the sedimentation coefficient of the major IgA peak

IgA Peak in 7S position

<u>Cyst fluid no.</u>	<u>Age</u>	<u>Menstrual status</u>	<u>Parity</u>
4	44	Not known	Not known
26	47	Postmenopausal	0
31	46	Hysterectomy	0
37	49	Postmenopausal	1
42	53	Not known	3
48	45	Premenopausal	3
57	38	Premenopausal	2
61	54	Not known	5
26*	54	Premenopausal	5

IgA Peak in 11S position

5 (2)	47	Premenopausal	3
8 (1)			
8 (2)	Not known	Not known	Not known
10 (1)	48	Premenopausal	0
10 (2)			
13 (2)	40	Premenopausal	2
18	49	Premenopausal	1
38 (1)	40	Premenopausal	0
44	32	Premenopausal	0
23*	42	Premenopausal	3

* Asterisks indicate cyst fluids obtained from Group II patients. All other cyst fluids were obtained from Group I patients.

The study of gross cystic disease of the breast is of particular importance because of the higher than expected incidence of breast carcinoma in patients with gross cystic disease (Warren, 1940; Lewison and Lyons, 1953; Veronesi and Pizzocara, 1968; Haagensen, 1971). However, the location of the malignant tumour is rarely related to the site of previous cysts, and it has been suggested that gross cystic disease and carcinoma of the breast share a common aetiologic factor (Haagensen, 1971). Histologically, gross cystic disease appears to be characterised by hyperplasia of acinar epithelium associated with dilatation of the terminal ducts (Schnug and Cavanagh, 1966), and it has been proposed that breast cyst fluid is a unique secretion from breast epithelial cells, possibly in response to abnormal hormone stimulation (Haagensen, Mazoujian, Dilley, Pedersen, Kister and Wells, 1979), with an intracellular-like ionic consistency (Fleisher, Robbins, Breed, Fracchia, Urban and Schwartz, 1973). Biochemical analysis of cyst fluid is at present incomplete: CEA (Fleisher, Oettgen, Breed, Robins, Pinsky and Schwartz, 1974), various enzymes (Schwartz, Fleisher, Breed, Ashikari, de Palo, Kinne and Urban, 1976), various steroid hormones (Bradlow, Fukushima, Rosenfeld, Boyer, Kream, Fleischer and Schwartz, 1976), protein hormones (Srivastava, Pescoritz, Singh, Perisutti and Knowles, 1977), oestriol conjugates (Raju, Ganguly and Levitz, 1977), a progesterone binding component (Pearlman, Gueriguian and Sawyer, 1973), albumin (Fleisher *et al.*, 1973), haemosiderin (Haagensen, 1971) and other proteins (Haagensen *et al.*, 1979) have all been found in cyst fluids, but the origin of the fluid within the cyst is still unresolved.

The results of this study show that a wide range of IgA, IgG, lysozyme, lactoferrin, albumin and DHAS concentrations can be detected

in cyst fluids. Only DHAS concentrations seemed to be affected by the prolonged storage period of up to two years at -20°C , as judged by mean levels of DHAS in Group I and Group II patients using Student's t-test. However, it is possible that this may not be the appropriate statistical test to use since protein and DHAS values may not be normally distributed (see below).

The rate of detection was probably a reflection of the sensitivity of the assay method used being 100% for the substances detected by RIA (IgA, DHAS) and less for the substances detected by radial immunodiffusion. The high detection rate for lactoferrin, which is not normally detectable in serum, suggests that the cyst fluid probably does indicate the synthetic activity of the surrounding breast tissue.

Density gradient ultracentrifugation was used to discover the cause of the large variations of IgA, IgG and albumin concentrations observed. Of nineteen cyst fluids analysed, half contained IgA that was wholly or predominantly of the 11S type, and the remaining half contained IgA of the 7S type. The latter cyst fluids also contained high concentrations of two other serum proteins: IgG and albumin. This suggested that components of serum were entering the cyst by some unknown route. Interestingly, these cyst fluids also contained low concentrations of DHAS, similar to concentrations in serum, suggesting that fluid was also possibly entering the cyst from the vascular compartment. The appearance of serum proteins in cyst fluid was not due to the method of aspiration as shown by repeated analysis of fluid from the same cyst after a 21 day interval.

The high concentrations of 11S IgA present in some cyst fluids suggested that local synthesis of 11S IgA was occurring. In these cyst

fluids, very high concentrations of DHAS were observed. These values were similar to the concentrations found in normal breast secretions (Miller, Humeniuk and Kelly, 1980). Unfortunately the occurrence of mixtures of 11S and 7S IgA in some cyst fluids indicated that no single IgA standard was suitable for IgA measurement.

Insufficient clinical data was present to draw any firm conclusions about the relationship between milk protein and DHAS concentrations, and the various clinical parameters recorded. The difference in menstrual status between patients whose cyst fluids contained mainly 7S IgA, and those whose cyst fluids contained mainly 11S IgA was intriguing. It may have been due to reduced synthesis of 11S IgA in the postmenopausal breast, so that the only way in which a cyst fluid from a postmenopausal patient could contain high IgA levels would be by entry of 7S IgA from the vascular compartment. Unfortunately, it was not possible to analyse a larger number of cyst fluids by ultracentrifugation for practical reasons, and this explanation therefore remains unproven.

The results of this study indicate that breast cysts may be more heterogenous than previously thought, and that two or more types of cysts may occur in gross cystic disease of the breast. It is unlikely that cyst fluid only reflects the synthetic activity of the surrounding breast tissue and further studies are required to discover if the determination of milk protein or DHAS concentrations for a particular cyst fluid is of any clinical importance to the patient. It is conceivable that the high concentrations of DHAS in some cyst fluids might act as a precursor reservoir for the synthesis of more powerful hormones such as testosterone as suggested by Miller et al. (1980). Alternatively, high 11S IgA concentrations in cyst fluid might indicate

that the particular breast from which the cyst fluid originates is functioning 'normally' and therefore less at risk of subsequent malignant change, than a breast with cysts containing fluid with high 7S IgA concentrations.

CHAPTER 7

BREAST FEEDING AND SERUM IGA

IN THE NEONATE

7.1 INTRODUCTION

A major function of the breast is the transfer of immunity from mother to neonate (Chandra 1978, McClelland et al., 1978). Colostrum and milk are rich in IgA and the breast fed infant ingests large amounts of this immunoglobulin. Neonatal serum, in contrast to maternal milk, is deficient in IgA, reflecting the immaturity of the IgA synthesising system at birth (Tomasi and Grey, 1972). As described previously, IgA in colostrum and milk consists largely of 11S IgA (Newcomb et al., 1968; Hurlimann, Waldesbuhl and Zuber, 1969) which is more resistant to digestion by intestinal proteolytic enzymes in the neonatal gut than 7S IgA, due to the presence of secretory component (Brown, Newcomb and Ishizaka, 1970). Although it is known that the majority of IgA in feeds of colostrum and milk is not absorbed by the infant (Ammann and Stiehm, 1966; Kenny, Boesman and Michaels, 1967; Ogra, Weingraub and Ogra, 1977), it has been suggested that the gut in early neonatal life is capable of absorbing small quantities of IgA (Iyengar and Selveraj, 1972; Ogra et al., 1977). However, the assay techniques which have been used have been relatively insensitive and it was considered desirable to examine this question using RIA, with its greater sensitivity, in a study of normally breast-fed infants.

The purpose of the study described in this chapter was to determine if exclusive breast feeding for one week altered IgA levels in the serum of neonates. IgA concentrations in the serum of exclusively breast-fed neonates were therefore compared with IgA levels in the serum of exclusively artificially fed neonates. In addition, the

metabolism of IgA in early life was studied by analysing cord blood samples and comparing IgA concentrations present at birth with those found in the same infant six days later. The molecular size of IgA in the neonate was also estimated by gel filtration.

7.2 MATERIALS AND METHODS

Sera: Forty-eight healthy, full term neonates were studied: 23 of the neonates were exclusively artificially fed and 25 were exclusively breast fed. Cord blood was collected from 24.

On the sixth day after delivery, a heel prick blood sample is routinely taken for Guthrie testing, and a further 0.5 ml blood sample was taken from the same site into a polythene capillary tube (Sarstedt). The blood was allowed to clot, the serum separated by centrifugation and stored at -20°C till analysis. All blood samples were collected by Dr. P.J. Latham, whose assistance is gratefully acknowledged.

Quantitation of serum IgA concentrations

IgA concentrations were determined by RIA as described in Chapter 4.

Gel filtration

Gel filtration studies were carried out using a Sepharose 4B (Pharmacia) column as described in Chapter 4. Immunoassay was performed on unconcentrated fractions.

Statistical methods

Student's t-test was used to compare the arithmetic means of serum IgA concentrations in the breast-fed and artificially fed neonates. A paired Student's t-test was used to compare cord blood samples with further serum samples taken from the same baby 6 days later.

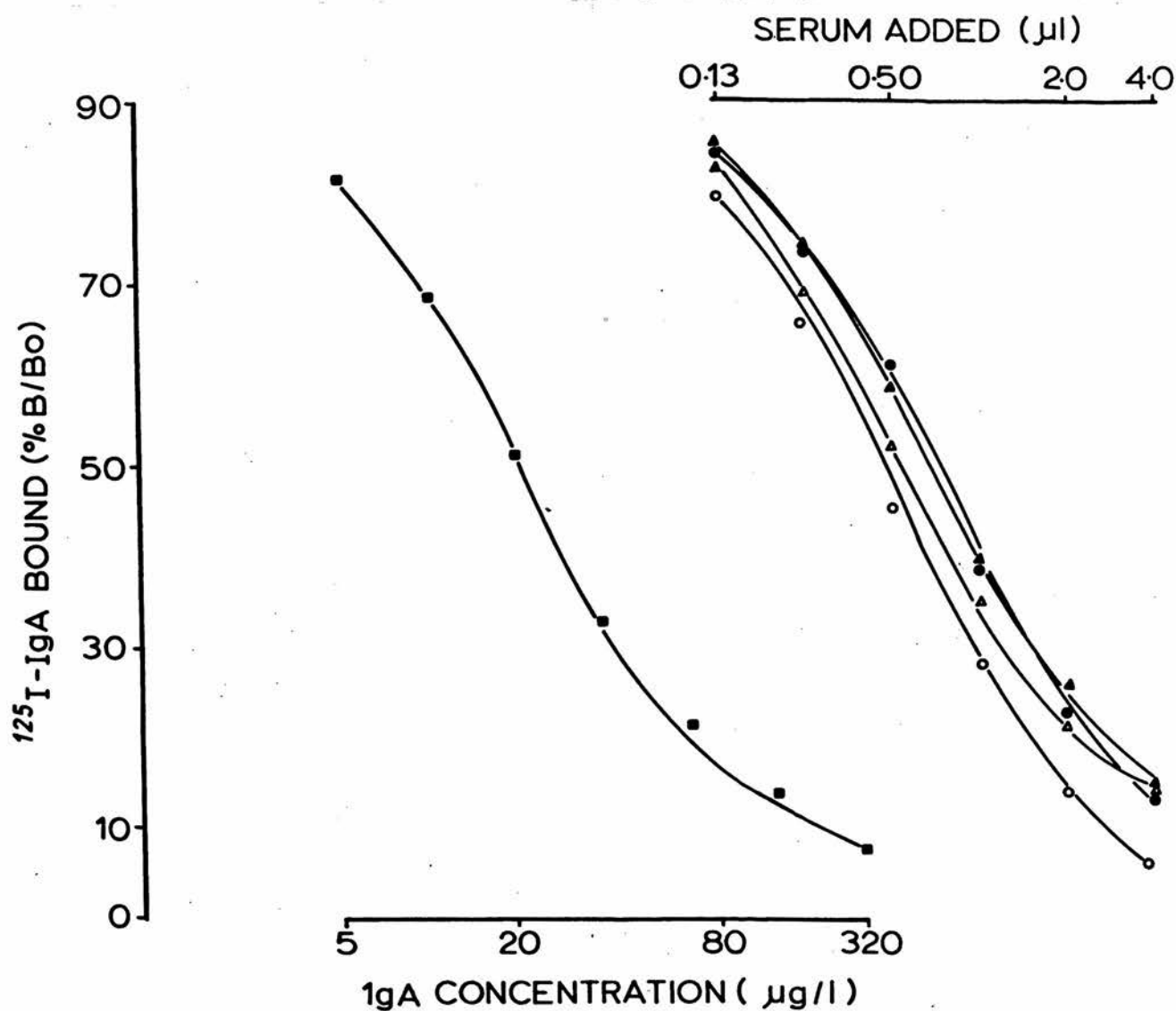


Fig. 7.1:

Inhibition curves for serum IgA standard (■—■), cord blood (▲—▲ and △—△), six-day heel prick serum from an exclusively artificially-fed neonate (○—○) and six-day heel prick serum from an exclusively breast-fed neonate (●—●).

7.3 RESULTS

Standard inhibition curve for IgA

The binding of ^{125}I -11S IgA to rabbit anti-7S IgA was inhibited in a parallel manner by the 7S IgA standard, two samples of cord blood, one sample of heel prick blood from a 6-day old neonate that had been artificially fed, and one sample of heel-prick blood from a 6-day old neonate that had been breast fed (Fig. 7.1). This indicated that immunoassayable IgA was present in cord blood and day 6 heel prick blood.

Characterisation of IgA in cord blood and day 6 serum

A single peak of immunoassayable IgA on Sepharose 4B gel filtration was found in cord blood, pooled sera from 4 artificially fed and 4 breast fed six-day old neonates, human colostrum, human milk and from adult serum (Fig. 7.2, 4.8 and 4.9). Cord blood and heel prick serum IgA eluted at the same volume as adult 7S IgA and at a different volume from 11S IgA present in milk and colostrum, indicating that the predominant form of IgA in both cord blood and day 6 sera is the 7S monomeric form. The trace of immunoassayable material found in infants' serum at the elution volume of secretory IgA was at most 2.5% of the 7S peak, suggesting that there is very little 11S IgA in day 6 serum. The 7S IgA standard was therefore used for all subsequent measurements of IgA in cord blood and serum.

IgA concentration in cord blood

The concentrations of IgA in 24 cord blood samples ranged from 0.52-516.0 mg/l. Four neonates had cord blood levels that were greater than 10 mg/l and in two of these neonates, IgA levels decreased from 516 mg/l to 0.33 mg/l and from 329 mg/l to 3.08 mg/l in 6 days (Fig. 7.3).

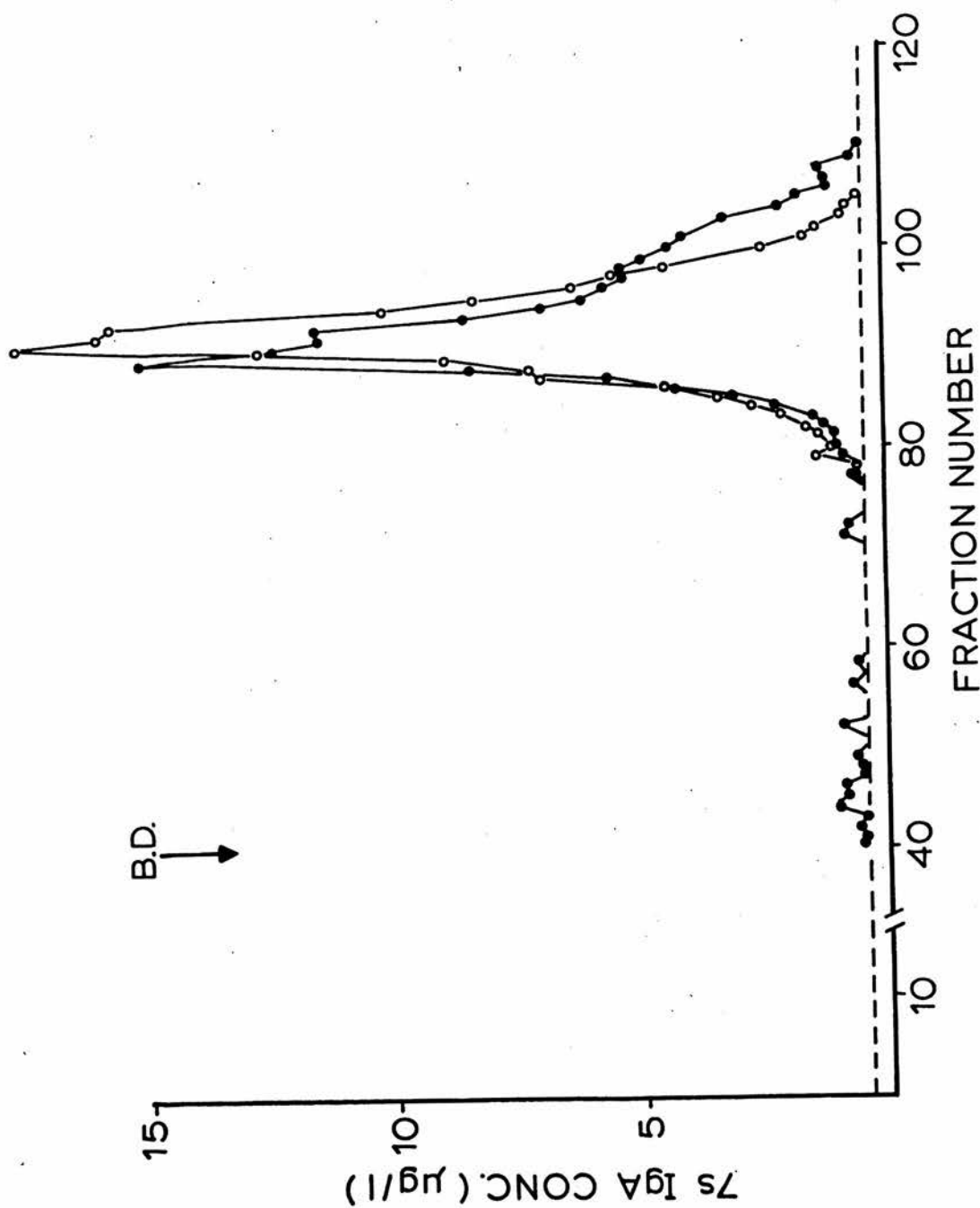


Fig. 7.2: Gel filtration on a Sepharose 4B column of cord blood (○—○) and pooled heel prick serum (●—●) from 8 six day old neonates: 4 artificially fed, 4 breast fed (cf. Fig. 4.8 and 4.9 where identical experimental conditions were used). 3 ml fractions were collected; the arrow indicates the elution volume of a Blue Dextran marker (B.D.; void volume), and the broken line the limit of detection in this particular assay.

A significant decrease in IgA concentrations occurred during the first 6 days of life in 20 neonates from whom both cord and day 6 sera were tested; this was found in both artificially and breast-fed neonates (paired t-test, $t = 3.09$, $P < 0.01$, Fig. 7.3). The four neonates with cord blood levels greater than 10 mg/l were excluded from statistical analysis because their cord sera were probably contaminated with maternal blood.

IgA concentrations in day 6 serum

Serum IgA levels in 23 exclusively artificially fed and 25 exclusively breast-fed babies on the sixth day of life were 2.71 ± 0.25 mg/l and 2.58 ± 0.33 mg/l (arithmetic mean \pm SE) respectively (Fig. 7.3). There was no significant difference between mean IgA concentrations in the two groups showing that exclusive breast feeding had no detectable influence on serum IgA levels after 6 days. One baby had less than 0.14 mg/l of IgA in its serum suggesting that it might be deficient in IgA. This baby also had the lowest IgA concentration in its cord blood.

7.4 DISCUSSION

In this chapter, the influence of breast feeding on IgA concentrations in the serum of neonates was studied as the neonate is IgA deficient at birth and breast feeding might therefore be able to compensate for this. In comparison, IgG concentrations in neonatal serum are equal to or greater than maternal serum IgG concentrations due to transplacental transfer of IgG but not IgA (McClelland and Samson, personal communication). The properties of IgA antibodies in serum have not been clearly delineated, due to the difficulties of obtaining IgA preparations devoid of contaminating antibodies of

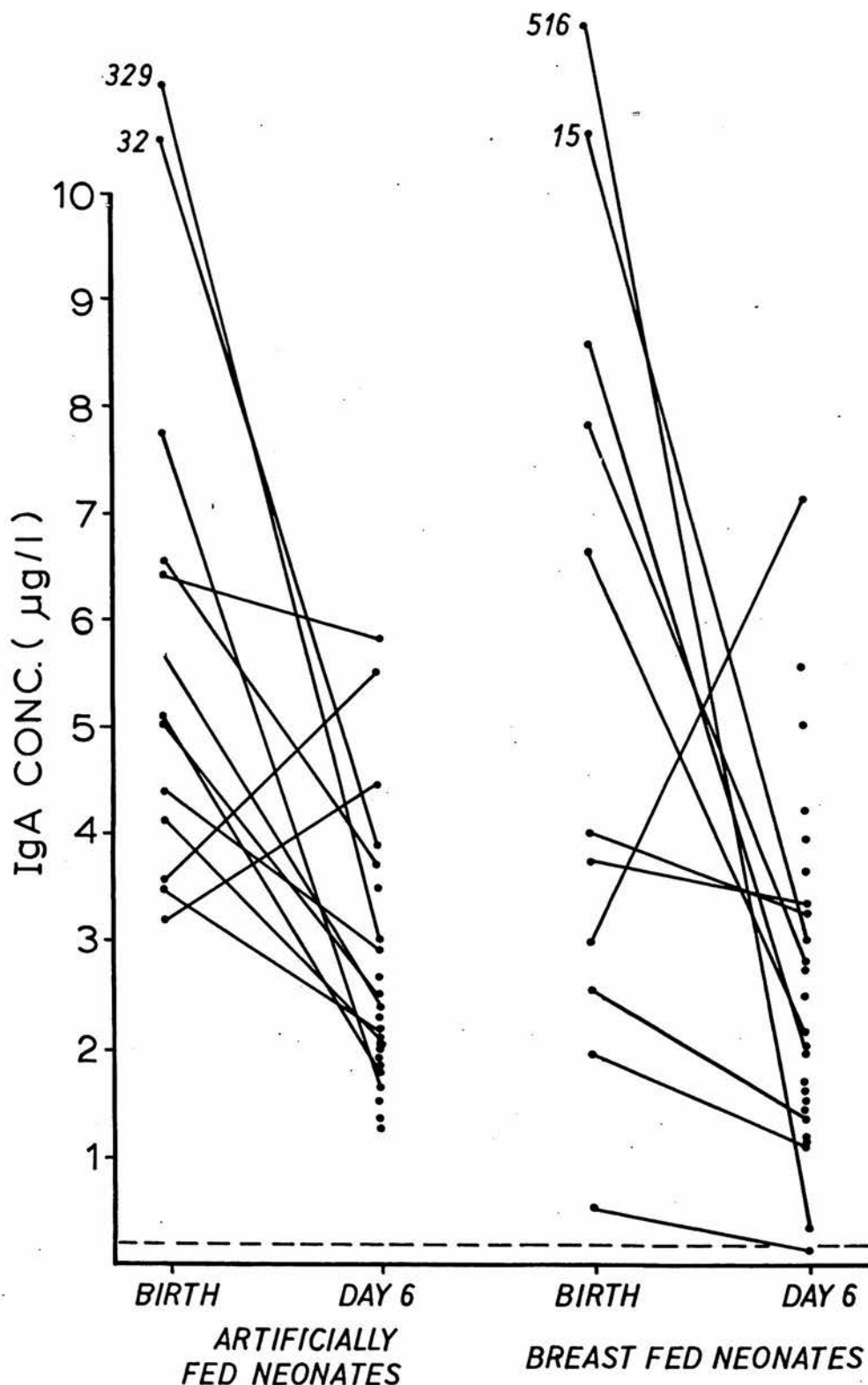


Fig. 7.3:

Serum IgA concentration in the cord blood of 24 newborn infants and heel prick sera of 23 artificially-fed and 25 exclusively breast-fed six-day old neonates. The solid lines indicate cord blood and heel prick sera that were collected from the same baby, and the broken line the limit of detection for serum IgA in this particular assay.

other immunoglobulin classes. Although IgA immunoglobulins cannot fix complement by the classical pathway, 7S IgA and 11S IgA have been reported to be effective activators of the alternative pathway if artificially aggregated (Gotze and Muller-Eberhard, 1971; Colten and Bienenstock, 1974). The ability of IgA antibodies to opsinize is controversial (for references, see Lamm, 1976), and it is thought that the most important function of IgA antibodies is not in serum but in external secretions e.g. gastrointestinal fluids, where 11S IgA antibodies can prevent bacteria from adhering to epithelial cells, thus limiting bacterial colonisation and penetration of mucous surfaces (Williams and Gibbons, 1972). In addition, it is likely that 11S IgA antibodies can prevent the absorption of foreign proteins by the formation of antigen-antibody complexes which are not absorbed (Walker, Isselbacher and Bloch, 1972; Andre, Bazin and Heremans, 1973).

In view of the IgA deficiency that exists at birth, breast feeding might be an important mechanism for the transfer of immunity to the serum compartment of the human neonate. In animal species where immunoglobulin transfer via the placenta does not occur (horses, pigs, cows, goats), absorption of colostral immunoglobulins (mainly IgG) from the gut of the newborn takes place (Brambell, 1970). In human neonates, the evidence for absorption of immunoglobulins from the neonatal gut is conflicting. Although large amounts of IgA are present in colostrum and breast milk (e.g. 3.52 gm/24 hr and 1.31 gm/24 hr of 11S IgA is produced on the second and fifth day post-partum, McClelland et al., 1978), Ammann and Stiehm (1966) found no difference in immune globulin levels between artificially-fed and breast-fed infants after 4 days of breast feeding. In contrast,

Iyengar and Selveraj (1972) reported significantly higher immunoglobulins on the fifth day of life in colostrum-fed infants compared with artificially-fed infants. Ogra et al. (1977) reported a rise in serum IgA levels in 3 infants who were fed colostrum 18 to 24 hours after birth and suggested that the gut is capable of absorbing IgA at this stage. In these studies IgA levels were determined by the relatively insensitive technique of single radial immunodiffusion which has a lower limit of detection of approximately 10 mg/l; Ogra et al. (1977) used the slightly more sensitive technique of radioimmunodiffusion.

In the studies described in this chapter, low concentrations of IgA (a thousand-fold less than the IgA concentrations seen in adult serum) were found in all the neonates studied by RIA, and these levels were not influenced by breast feeding. Furthermore, it was found that in infants' sera, less than 2.5% of the immunoreactive IgA-like material behaves like 11S IgA on gel filtration. Breast feeding would have been expected to produce an immunoreactive peak at the 11S position in neonatal sera if the 11S molecule was absorbed in the intact form by the neonatal gut.

The data demonstrates firstly, that IgA absorption had ceased by the time of sampling at the sixth day, and secondly, that if there was a period of IgA absorption before the sixth day, IgA entering the circulation must have been cleared with a half-life value considerably less than the adult serum IgA half-life value of approximately 5 days (Waldmann and Strober, 1969). The collection of blood was delayed till the sixth day post-partum because it was considered unethical to obtain samples earlier, as no routine samples were being taken at that time.

The values for cord blood concentrations of IgA observed are consistent with the concentrations found by other workers using techniques of similar sensitivity (Faulkner and Borella, 1970; Schuurman, Haagenars and Zegers, 1977). However, 4 neonates were found to have cord blood concentrations that were greater than 10 mg/l. When these 4 neonates were studied 6 days later, their respective serum IgA levels had decreased much more than would be expected if the half-life of serum IgA in the infant is similar to that in the adult. It is therefore likely that these high values may be due to contamination of cord blood with maternal blood at the time of collection. Earlier reports of raised IgA in cord blood serum (Stiehm, Ammann and Cherry, 1966) may therefore have been due to contamination of cord blood by maternal blood (with thousand fold higher IgA concentrations) at the time of collection.

In the remaining 20 neonates in whom paired cord and day 6 sera were collected, a significant decrease in serum IgA was observed in the first 6 days of life. It is possible that this may have been due to transplacental transfer of maternal IgA or to minimal contamination with maternal blood at the time of cord blood collection. It was unfortunately not possible to distinguish between these two possibilities with the methodology available. It is unlikely that this decrease is due to cord material interfering with a sensitive inhibition test as parallelism in an inhibition curve was observed between IgA in adult serum and IgA in cord blood. In addition, only a single peak of immunoreactivity (at the elution volume of adult 7S IgA) was found in cord blood on examination by gel filtration. Any substance that interfered with cord blood IgA determinations would have

been expected to elute at a different volume on gel filtration to 7S IgA, and therefore would have been detected on immunoassay of the gel filtration fractions.

It was not possible to detect IgA at the assay dilution used in one neonate, indicating a level less than 0.14 mg/l. This neonate also had the lowest cord blood value among all neonates studied. Unfortunately, there was insufficient serum for further analysis using more sensitive conditions for IgA detection but it is possible that this infant may be selectively deficient in IgA. This condition has not been previously reported in neonates, but further studies of this neonate when he is older are required for confirmation.

In conclusion, it is likely that the principle physiological of colostrum and breast milk IgA in the neonate is at the level of the intestinal lumen or mucosal surfaces (Brambell, 1970) as no detectable transfer of IgA into the circulation of infants occurs.

CHAPTER 8

GENERAL DISCUSSION

CHAPTER 8 GENERAL DISCUSSION

The aim of the experiments described in this thesis was to study the human mammary secretory immune system in different reproductive states by measuring 11S IgA secretion. Most of the studies on this subject have been concerned only with the post-partum lactating breast, and the experiments described in this thesis have extended knowledge about mammary secretory immunity to the neonate and the non-lactating woman, and have also demonstrated the influence of prolactin on the development and maintenance of secretory immunity in the breast. In this final chapter, the experimental observations described in this thesis are related to current views of the development and control of secretory immunity outwith the breast. The relevance of these observations to the aetiology of carcinoma of the breast is also discussed.

The origin of the precursors of IgA secreting lymphocytes

In the human, the precursor lymphocytes destined to differentiate into the antibody synthesizing cells of the secretory immune system probably originate in the foetal liver (Lawton, Self, Royal and Cooper, 1972) or bone marrow (Abdou and Abdou, 1972). From there, these lymphocytes then migrate to gut-associated lymphoid tissue (GALT) consisting mainly of collections of lymphoid tissue in the submucosa of the small intestine known as Peyer's patches. While in the liver or bone marrow, these lymphocytes are influenced in an unknown manner, so that they become committed to antibody synthesis (Lamm, 1976). In the chicken, this commitment takes place in the Bursa of Fabricius, and these lymphocytes destined to synthesize antibody are therefore called B lymphocytes (bursa equivalent derived lymphocytes).

Other lymphocytes migrate from the foetal liver or bone marrow instead to the thymus where their development is influenced in a different manner, so that they subsequently participate in cell mediated immunity, or in the control of antibody synthesis by B lymphocytes. Some thymus derived lymphocytes (T lymphocytes) may either suppress antibody synthesis (T suppressor lymphocytes) or stimulate it (T helper lymphocytes).

The role of antigen in the development of the secretory immune system

The access of GALT to antigens within the intestinal lumen is thought to be important in the activation of IgA precursor lymphocytes in the secretory immune system. Cellular proliferation and the attainment of the full size of Peyer's patches is highly antigen dependant, and in germ-free mice, both IgA plasma cells in the intestines and IgA production are decreased (Stecher and Thorbecke, 1967; Crabbe, Bazin, Eyssen and Heremans, 1968; Vitetta, Grundke-Iqbal, Holmes and Uhr, 1974).

The access of GALT to intestinal antigen is thought to be assisted by a specialised epithelium overlying the lymphoid tissues (Bockman and Cooper, 1973). The cells of this epithelium were reported by Owen (1977) to preferentially take up horseradish peroxidase after exposure to small quantities of this antigen, and then to release the antigen into the underlying interstitial space for processing by the lymphoid cells within Peyer's patches. Interestingly, in the same experiments, a generalised uptake by other non-specialised epithelial cells was noted at higher concentrations of horseradish peroxidase, suggesting that the mode of access to GALT may be dependant upon antigen concentration within the intestinal lumen.

The role of the thymus

Although B lymphocytes are responsible for the synthesis of the large quantities of IgA appearing in external secretions, a number of studies have emphasised the importance of the thymus, or of T lymphocytes in the development of the secretory immune system. Firstly, Peyer's patches in congenitally athymic ('nude') mice do not contain germinal centres, and although IgA bearing lymphocytes are observed in the peripheral blood of such animals, IgA plasma cells in the lamina propria of the gut (considered to be part of the secretory immune system) are markedly reduced. Furthermore, these mice do not respond to antigenic challenge with a normal rise of IgA antibody (Hauptman and Tomasi, 1978).

Secondly, in humans, thymic abnormalities are often associated with deficiencies of IgA. In ataxia-teleangiectasia, there is a reduction in salivary 11S IgA as well as serum IgA despite the fact that these patients have normal numbers of circulating B lymphocytes bearing IgA (Peterson, Kelly and Good, 1964). This suggests that there is either a lack of specific IgA T helper lymphocyte activity, or alternatively, a relative excess of T suppressor lymphocytes specific for IgA. The latter defect has been demonstrated in IgA deficient patients, many of whom have associated defects in secretory IgA (Waldman et al., 1974).

Thirdly, the elegant experiments of Elson, Heck and Strober (1979) have demonstrated variations in regulatory T lymphocyte activity among various murine lymphoid tissues for IgA, but not IgG or IgM. Peyer's patches were found to contain a high level of IgA T lymphocyte helper activity in contrast to peripheral or mesenteric

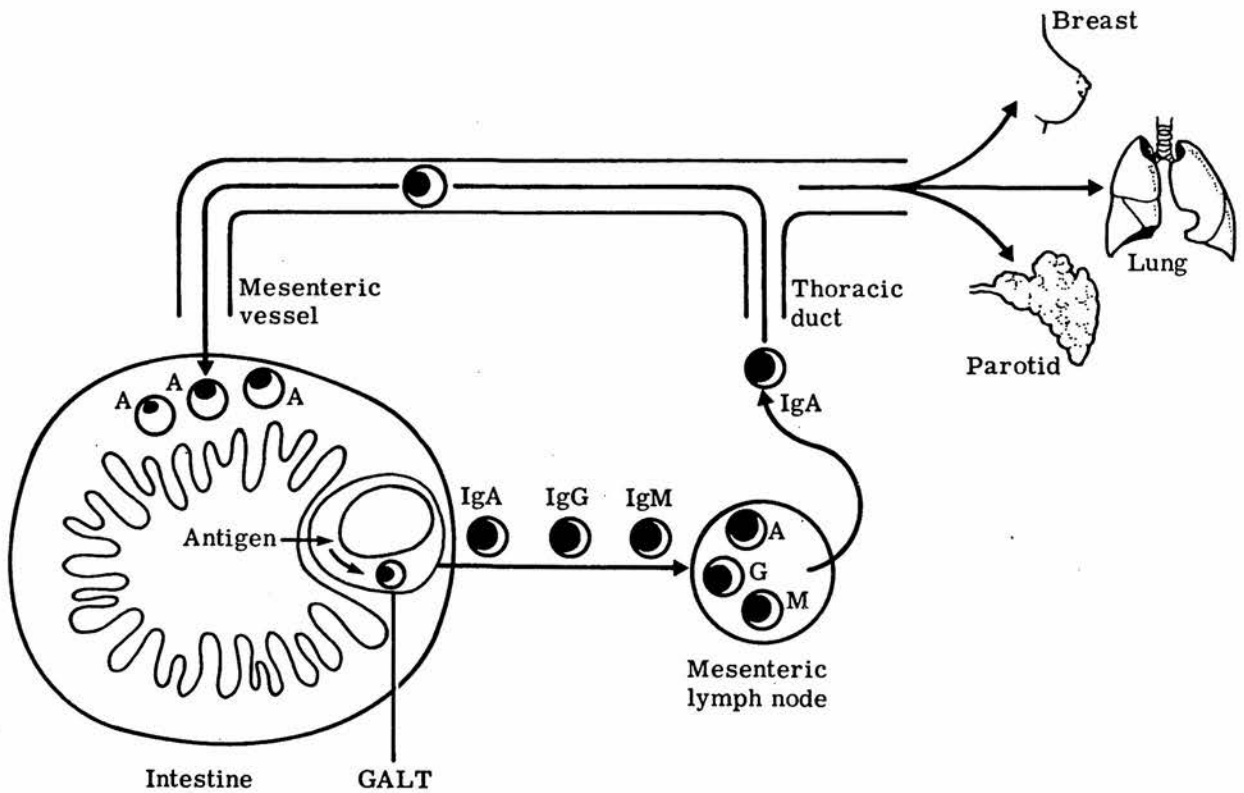


Fig. 8.1:

A diagrammatic representation of the movement of lymphocytes from Peyer's patches to sites of secretory immunity.

Antigen from the intestinal lumen activates B lymphocytes in Peyer's patches (part of the gut-associated lymphoid tissue, GALT), and IgA precursor lymphocytes pass sequentially through the mesenteric lymph nodes to sites of secretory immunity (e.g. the breast, lung, salivary gland and the lamina propria of the gut), where they differentiate into IgA secreting plasma cells. It is thought that IgA precursor lymphocytes do not return to Peyer's patches but 'home' to the adjacent lamina propria.

lymph nodes and the spleen. In all the above lymphoid tissues, T lymphocyte suppressor activity was found for IgG and IgM leading this group to propose that in the mouse, a specific subset of helper T lymphocytes is involved in the regulation of IgA synthesis.

Further indirect evidence supporting the above concept arises from the observation that GALT cells, when isolated and incubated in vitro, do not mature spontaneously into plasma cells capable of synthesizing antibody, suggesting that helper T lymphocytes and perhaps other factors like bacterial mitogens (e.g. lipopolysaccharide) may be required for maturation of precursor lymphocytes into IgA plasma cells (Walker and Isselbacher, 1977).

The entero-mammary lymphocyte cycle

Extensive evidence for the circulation of IgA precursor lymphocytes from GALT to the mammary gland exists, as described in Chapter 1. It is currently thought that following activation by intestinal antigen, precursor lymphocytes pass sequentially through the mesenteric lymph nodes, the thoracic duct, the blood vessels, finally reaching the mammary gland and other sites of secretory immunity (reviewed by Lamm, 1976; Fig. 8.1).

This movement of lymphocytes from GALT to sites of secretory immunity has been described as 'homing' a term which implies that a directed migration of precursor IgA lymphocytes exists. The exact factor(s) that control homing are unknown, but one factor that has been proposed is secretory component. This protein is synthesized by epithelial cells, and binds preferentially to polymeric rather than monomeric IgA (Radl, Klein, van den Berg, de Bruyn and Hijmans, 1971). Furthermore, it has been demonstrated that a tendency exists for IgA plasma cells in different locations to bind secretory component

(Brandtzaeg, 1973). However, it should be noted that no direct evidence for the interaction of plasma cell surface IgA and locally produced secretory component exists. Furthermore, IgA precursor plasma cells arriving in a mucous membrane do not directly impinge on the epithelial cells, making such an interaction less likely, and experiments conducted by McWilliams, Philips-Quagliata and Lamm (1975) in mice consisting of passively raising IgA levels or injecting antiserum to secretory component failed to disrupt this hypothetical interaction.

Another possibility, first proposed by Gowans and Knight (1964), is that homing (to the lamina propria of the gut) might reflect a specific attraction or retention of cells that had been previously sensitised to intestinal antigens. In support of this, IgA producing plasma cells (presumably capable of synthesizing cholera antitoxin) were demonstrated to home to the small intestine after oral challenge with the toxin (Pierce and Gowans, 1975). However, against this hypothesis, Griscelli, Vassali and McCluskey (1969) demonstrated that homing of mesenteric node lymphoblasts to the gut occurs in germ free animals which are antigen deficient. Similar homing of thoracic lymphoblasts to the small intestine of unsuckled neonatal animals delivered by Caesarian section also occurs (Halstead and Hall, 1972) as does lymphocyte homing to grafts of foetal intestine explanted to sites free of antigen exposure (Moore and Hall, 1972; Parrott and Ferguson, 1974). In the latter study, the homing lymphocytes were capable of differentiating into plasma cells, secreting IgA into the lumen of the gut explant. Thus it is likely that lymphocyte homing is independent of antigens and it is difficult to see how such a mechanism might operate in the mammary gland.

The requirement for IgA precursor lymphocytes to be specifically involved in homing has recently been questioned by Brandtzaeg ^{et al} et al. (1979). In IgA deficient individuals, it is known that there are compensatory increases of IgM B lymphocytes in the intestine, and of IgD B lymphocytes in the upper aero-digestive tract. Elson et al. (1979) proposed an alternative hypothesis to homing, based on their studies of murine IgA synthesis described above. This group proposed that IgA B lymphocytes randomly distribute throughout the body after activation by intestinal antigen, but lodge and terminally differentiate only in sites where there is sufficient IgA T helper lymphocyte activity. This proposal, although attractive, raises the problem of how a differential distribution of T helper and suppressor activity in the different lymphoid tissues is achieved, and perhaps the homing of T lymphocytes to the gut described by various workers (Sprent and Miller, 1972; Guy-Grand, Griscelli and Vasalli, 1974; McWilliams et al., 1975) is of significance in understanding the control of secretory immunity.

In view of the concepts discussed above, the observations described in this thesis on the neonatal mammary gland would be consistent with activation of neonatal GALT by intestinal antigen following parturition, followed by a rapid (within days) influx of IgA precursor lymphocytes into the neonatal mammary gland, or alternatively, an alteration in the secretory activity of the IgA lymphocytes present in the mammary gland. Current evidence (as described above) would favour the former, but further experiments might be indicated to assess the number and class of lymphocytes present in the neonatal mammary gland pre- and post-partum, and to determine the specificity of the IgA antibody present in neonatal milk to see if it is directed

against intestinal antigens.

The Entero-Mammary Cycle and Immunological Protection of the Neonate

The enter^o-mammary circulation of lymphocytes described in the preceding pages is of great significance to the immunity of the neonate, since it results in the 11S IgA antibody content of colostrum and milk reflecting the antigenic exposure of the maternal gut. The gut of the breast fed neonate is therefore provided with colostrum or milk antibodies against microorganisms harboured by, or passing through the maternal intestine, and also against micro-organisms present in the environment and likely to reach the neonate. It is worth noting that in some animals, coprophagia by the mother of the newborn's faeces can be observed e.g. the rat, and in other animals licking of the hindquarters of the newborn by the mother is seen e.g. the deer. If gastro-intestinal infection in the newborn animal occurs, this behaviour is thought to allow the rapid production of specific colostrum and milk antibodies to the offending organism via the ingestion of the organism, activation of GALT, seeding of the mammary gland with IgA precursor lymphocytes and local (intra-mammary) synthesis of the 11S IgA antibody. Although this newborn/maternal cycle is unlikely to operate in humans, these observations may suggest that the current concern with sterility in the care of the neonate may not be warranted, at least in the case of the breast fed neonate.

It may be possible to utilise the entero-mammary cycle for therapeutic benefit by specifically immunising the lactating mother by intestinal exposure to the relevant antigens. This possibility has been demonstrated by Svennerholm, Holmgren, Hanson, Lindblad,

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Quereshi and Rahimtoola (1980) who showed that in lactating Pakistani mothers, a rise in the 11S IgA antibodies in milk against *V. cholerae* could be achieved by parenteral immunisation (vaccination) following natural intestinal exposure to *V. cholerae*. This manipulation of the secretory immune system coupled with breast feeding may be of great value in countries with high infant mortality rates due to gastroenteritis.

The role of prolactin

It has previously been suggested that the ability to transport secretory immunoglobulins e.g. 11S IgA from the sub-mucosal tissue fluid to the luminal side of mucosae is a general property of epithelium developmentally derived from the gut (Hall, Orlans, Peppard and Reynolds, 1977). This would include all the known sites of secretory immunity (the alimentary tract, its associated glands, the liver, the nasopharynx, the upper respiratory tract and part of the genitourinary tract) except the mammary gland^{and lacrimal gland}, which arises as an appendage of the skin, and from ectoderm respectively.

The studies of Weisz-Carrington et al. (1978) clearly demonstrate that prolactin is the main hormone influencing the transit of IgA secreting lymphocytes to the murine mammary gland, and the studies in this thesis indicate that prolactin probably has a similar role in humans, in view of the high concentrations of 11S IgA in the mammary secretions of women with hyperprolactinaemic galactorrhoea.

In Chapter 1 of this thesis, the origin of the entire epithelium of the mammary gland from the small number of epithelial cells that represent the embryological milk line in the foetus is

described. If it is assumed that mammary epithelial cells possess some (unknown) property that influences the 'homing' of IgA precursor lymphocytes (Lamm, 1976), then it might be reasonable to suggest that the sensitivity of mammary epithelial cells to the action of prolactin (McNeilly, 1977) allows the mammary gland to possess a fully functional secretory immune system at the time of parturition, and shortly after, when prolactin levels are highest (Hwang et al., 1971). Thus the difference in ontogeny between the mammary gland and other sites of secretory immunity (Hall et al., 1977) might reflect the fact that the mammary secretory immune system is normally only functional post-partum, unlike other sites of secretory immunity which are functional continuously e.g. the gut.

In this thesis, high concentrations of 11S IgA were demonstrated in normal breast secretions, although the total quantity of 11S IgA present is much less than the amount present in normal post-partum milk or colostrum, or in galactorrhoeic mammary secretions, since the volume of normal breast secretions was much less. The high concentrations of 11S IgA in the normal breast secretions of premenopausal as well as postmenopausal women is consistent with the proposal that prolactin is the hormone indirectly responsible for the development and maintenance of the mammary secretory immune system, by the stimulation of epithelial proliferation. This is because peripheral blood levels of prolactin in non-lactating women during the menstrual cycle, and in postmenopausal women, are probably well in excess of threshold requirements for its action (McNeilly and Chard, 1974). Furthermore, prolactin concentrations in neonatal blood are very similar to the prolactin concentrations in maternal

blood post-partum, and decline in the same manner (Hiba et al., 1977) lending weight to the suggestion that prolactin might be influencing the neonatal mammary gland in the same way that it influences the post-partum maternal mammary gland.

The Mammary Turnover of IgA secreting lymphocytes

In discussing the mammary secretory immune system, the life span of IgA secreting plasma cells in the breast needs to be considered. Unfortunately, in the human, this is not known, but in the mouse, a value of 7 to 8 weeks for intestinal IgA plasma cells, and splenic IgG, IgA and IgM plasma cells has been reported (Mattioli and Tomasi, 1973). As the IgA secreting lymphocytes of the mammary gland originate from the gut, this would indicate that a regular replenishment of these lymphocytes must occur, and this concept is in agreement with the observation that immunological memory does not exist in the secretory immune system (Ogra and Karzon, 1969; Clough, Zaccari and Strober, 1970; Andre, Bazin and Heremans, 1973).

The presence of 11S IgA in normal breast secretions would therefore also indicate that an influx of IgA precursor lymphocytes must be occurring continuously into the non-lactating breast. This would be in agreement with the data of Drife et al. (1977), who demonstrated a significance difference in mammary IgA synthesis between the luteal and proliferative phase of the menstrual cycle of parous women, indicating that there must have been an increased influx of plasma cells into the breast at around the beginning of the luteal phase. The alternative possibility that this difference reflects an alteration in the IgA synthetic rate due to a change in the hormonal environment is less likely, since no such difference was detected in the biopsies from nulliparous women.

It is not possible to comment about the significance of the results of the cyst fluid analysis as the results were heterogenous and indicate that the hypothesis that cyst fluid might exclusively reflect the synthetic activity of the surrounding mammary tissue is probably too simplistic.

The Relevance of Experimental Data to Carcinoma of the Breast

To assess the relevance of the studies described in this thesis to carcinoma of the breast, one has to consider whether the observations described explain the protective effect of an early first pregnancy and, in addition, whether the 11S IgA antibodies detected have any functional role in the mammary gland.

It is clear that no qualitative changes are present in the mammary secretory immune system prior to the first pregnancy, since 11S IgA could be detected in the mammary secretions of the neonate and the nulliparous woman. In particular, assay methods were developed to confirm that it was the complete 11S IgA molecule that was being detected, and not the 7S IgA or 10S IgA molecule. There was therefore no evidence of defective IgA synthesis prior to the first pregnancy. However, it is possible that a quantitative alteration in IgA synthesis might occur following the first full-term pregnancy. Unfortunately, this hypothesis could not be tested because of the small number of normal breast secretions available for study, but it might be of value to carry out 11S IgA estimations on the breast secretions of a larger group of premenopausal women. It might also be worthwhile to confirm that all the normal breast secretions contained 11S IgA in view of the finding that cyst fluids contained 7S IgA, 11S IgA or both.

One disadvantage of the present method of studying breast secretions is the fact that ^{the} sample obtained is indicative only of immunoglobulin at or near the nipple, and may not reflect synthetic activity at the alveolar level. In order to study this problem further, it might be necessary to immunise a non-lactating woman orally to an appropriate antigen, and to assess the time interval before the appearance of specific 11S IgA antibody in the breast secretion.

In considering the relevance of these studies to carcinoma of the breast, it must be emphasised that 11S IgA in this thesis was measured as class-specific antibody, and no attempts were made to determine the nature of the antigens that the 11S IgA was directed against. Human colostrum and milk contain antibody activity against ABO blood group antigens, *E. coli*, *Staph. aureus*, a host of viruses (Coxsackie B5, Herpes simplex, poliovirus, rotavirus), cholera toxin and pneumococcus (reviewed by Butler, 1979), but it is not known what antibody activities are present in normal breast secretions in non-lactating women. Such information might be helpful in understanding the mode of formation of the secretions, and for formulating a role (if any) for breast secretions in the non-lactating breast.

It is conceivable that the presence of immunoglobulins in breast secretions outwith post-partum lactation simply reflects a low level of mammary epithelial proliferation resulting from low levels of serum prolactin, 'attracting' IgA precursor lymphocytes into the breast.

If immunotherapy for breast cancer consisting of the delivery of tumour specific IgA antibodies to the breast is ever considered,

then it may be appropriate to carry out oral immunisation with tumour antigen (to activate the appropriate GALT) at the same time as raising serum prolactin levels to stimulate mammary epithelial proliferation and the 'attraction' of the maximum number of IgA precursor lymphocytes into the breast.

Finally, it is possible that immunosurveillance (Burnet, 1965) of the mammary gland for carcinoma may not exist, and it is interesting to note that the most recent study of patients treated with immunosuppressive drugs demonstrated a striking excess of non-Hodgkin's lymphomas and to a lesser extent skin cancer, but no excess of breast cancers (Kinlen, Shiel, Peto and Doll, 1979). However, the studies in this thesis demonstrate that IgA B lymphocytes enter the mammary gland throughout life and further studies are required to assign a role to this process.

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PUBLICATIONS

The following papers were published on the basis of the experimental studies described in this thesis:

- Yap, P.L., Pryde, A. and McClelland, D.B.L. (1979) Serum IgA in the neonate: molecular size, concentration and effect of breast feeding. *Acta Paediatr. Scand.* 68, 695-700.
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